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Genotoxic effects in Cancer Patients after Treatment with Single Drug Etoposide or Melphalan Chemotherapy

by

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Diploma in Physics, Moscow State University, Moscow, Russia, 1992

A Dissertation Submitted in Partial Fulfillment of the
Requirements for the Degree of

DOCTOR OF PHILOSOPHY

in the Department of Biology

We accept this dissertation as conforming
to the required standard

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ABSTRACT

One of the complications of cancer chemotherapy is an increased risk of secondary treatment-related malignancy. It is believed that secondary cancers arise as a result of mutagenic and genotoxic effects of chemotherapeutic agents. The mechanisms by which these agents induce secondary cancer are not known. This study was designed to investigate the potential genotoxic effects of two agents, etoposide and melphalan, in cancer patients receiving single drug chemotherapy. Both drugs are potent mutagens and have been implicated as causative agents of secondary acute myeloid leukemia (sAML). Two groups of patients were studied: small cell lung cancer (SCLC) patients who received etoposide and multiple myeloma (MM) patients treated with melphalan. The induction of mutation at the hypoxanthine phosphoribosyl transferase (*hprt*) gene was investigated in patients' lymphocytes by using the *hprt* T-cell clonal assay. The results showed that the mean *hprt* mutant frequency (MF) did not increase after treatment with neither agent, however, a significant elevation of MF was detected in a patient who received the highest melphalan dose. The sequence analysis of mutants revealed a significant enhancement of AT>TA transversions in post-etoposide spectra compared to the control spectra, however, no mechanistic explanation for the induction of such mutation by etoposide can be proposed. Etoposide, a topoisomerase II inhibitor and an inducer of DNA strand breaks, is expected to produce large genomic deletions and rearrangements. No enhancement of large deletions or rearrangements was seen in post-etoposide spectra. The elimination of mutated cells by apoptosis is proposed to explain the negative result. In post-melphalan spectra, a significant enhancement of GC>TA

transversions was detected compared to the control spectra. GC>TA transversion is an expected melphalan-induced mutation since alkylating agent melphalan preferentially causes guanine adducts and crosslinks.

The results suggest that while single drug chemotherapy does not induce mutation in the majority of patients, exposure to high doses of chemotherapy agents may be associated with the induction of mutation. This correlates with the dose-dependent increase in the risk of secondary malignancy after chemotherapy. The significance of drug-specific changes in the mutational spectra seen in patients' lymphocytes can be evaluated after mutations in proto-oncogenes and/or tumor suppressor genes are identified in secondary cancer cases. Results also suggest that the *hprt* mutational spectrum might be a more sensitive biomarker of the genotoxic exposure than the *hprt* MF.

To investigate the clastogenic effect of etoposide chemotherapy, blood cells from SCLC patients were screened for the appearance of multiple lineage leukemia (*MLL*) gene rearrangements using fluorescent *in situ* hybridization (FISH). *MLL* gene is rearranged in more than 50% of sAML cases in patients previously treated with etoposide or related compounds. Analysis of the results revealed that FISH is not sensitive enough method for the detection of rare rearrangements. The use of RT-PCR technique is proposed as a more realistic approach for monitoring patients treated with etoposide chemotherapy for the appearance of the MLL gene rearrangements.

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To my parents, my sister and my husband Michael

ABBREVIATIONS

(s)AML - (secondary) acute myeloid leukemia

Topo II - topoisomerase II

MLL - mixed lineage leukemia

h(a)prt - hypoxanthine (adenine) phosphoribosyl transferase

(N)SCLC- (non-) small cell lung cancer

MNC - mononuclear cell

PHA - phytohaemoglutinin

IL - interleukin

CBS - calf bovine serum

CE - cloning efficiency

MF - mutant frequency

RT-PCR - reverse transcription- polymerase chain reaction

bp - base pair

MM - multiple myeloma

RFLP- restriction fragment length polymorphism

TCR- T-cell receptor

RR- relative risk

SCE- sister chromatid exchange

ATP(ADP)- adenosine tri(di)phosphate

DNA(RNA)- (deoxy)ribonucleic acid

m-AMSA- 4-(acridinylamino)methansulphon-*m*-anisidide

MDS- myelodysplastic syndrome

MAR(SAR)- matrix (scaffold) associated region

SDS- sodium dodecyl sulfate

CHO- Chinese hamster ovary

a(t)k- adenosine (thymidine) kinase

NHL- non-Hodgkin's lymphoma

PUVA- psoralen plus UV-A light

GSH- glutathione

B-CLL- B-cell chronic lymphocytic leukemia

BER- base pair excision repair

NER- nucleotide excision repair

ANPG- alkyl-N-purine DNA glycosylase

MMS- methyl methanesulfonate

BPDE- benzo[a]pyrene diolepoxide

PAH- polycyclic aromatic hydrocarbons

MDR- multidrug resistance

MRP- multidrug resistance protein

wt- wild type

PRPP- phosphoribosyl pyrophosphate

bcr- breakpoint cluster region

FISH- fluorescent *in situ* hybridization

Mo-MLV- moloney murine leukemia virus

trx- trithorax

YAC- yeast artificial chromosome

6-TG- 6-thioguanine

ENU- ethylnitrosourea

ICR-191- 2-methoxy-6-chloro-9[3-(2-chloroethyl)amino-propyl-amino]dihydrochloride

MNNG- *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine

PRPP- 5'-phosphoribosyl-1-pyrophosphate

IMP- idenosine 5'-monophosphate

GMP- guanosine 5'-monophosphate

mRNA- messenger RNA

RIT- radioimmunoglobulin therapy

SSCP- single-stranded conformation polymorphism

Vf- variant frequency

RB- retinoblastoma

CRR- cystein-rich region

EDTA- ethylenediaminetetracetic acid

SC(U)- splice characterized (uncharacterized)

E - exon

I- intron

D- deletion

F- frameshift

IS- insertion

TS (TV)- transition (transversion)

RARA- retinoid acid α–receptor

CHAPTER I. BACKGROUND AND INTRODUCTION

1. Secondary Treatment-Related Cancer: Risks and Chromosomal Abnormalities.

Long term survivors of cancer therapy are at the increased risk of developing secondary malignancy. The incidence and type of secondary malignancy depend on primary tumor type, treatment regimen (radiation and/or chemotherapy agents and dose scheduling), genetic predisposition, age and sex of the patients (Braekeller *et al.*, 1986, Curtis *et al.*, 1984). The most common type of secondary malignancy is leukemia, particularly acute myeloid leukemia (sAML). Patients who received chemotherapy for their initial treatment were shown to have a significantly elevated risk of sAML (RR=4.5, the relative risk (RR) of leukemia estimated as a ratio of observed *vs.* expected cases). Initial treatment with chemotherapy and radiation increased this risk to sevenfold (RR=7.4), while radiation alone increased the sAML risk to twofold (RR=2.5) (Curtis *et al.*, 1984). The use of multiple drugs in the treatment regimen was shown to have a synergistic effect on the sAML risks (Pedersen-Bjergaard *et al.*, 1994). Significant excess risk was observed following chemotherapy for breast cancer (RR=8.1), ovarian cancer (RR=22.2, reported range 22-100), and multiple myeloma (RR=9.5, reported range 100-1,000) (Curtis *et al.*, 1984). Other sites of primary cancer for patients at a higher risk of sAML include lung cancer, Hodgkin's disease, non-Hodgkin's lymphoma and leukemia. Treatment of nonneoplastic conditions with chemotherapy agents or radiation was also associated with increased leukemia risk (Curtis *et al.*, 1984, Pedersen-Bjergaard *et al.*, 1994). The excess incidence of sAML begins about 2 years after exposure and increases

by 0.5% to 1% per year for at least 6-9 years (Curtis *et al.*, 1984, Pedersen-Bjergaard *et al.*, 1994). The specificity of secondary leukemia is related to the treatment regimen used.

Alkylating agents such as nitrogen mustards (cyclophosphamide and melphalan) and drugs targeting topoisomerase II (Topo II) such as epipodophyllotoxins (etoposide, teniposide) and antracyclines are among the most leukemogenic currently used anticancer agents. There are two distinct forms of sAML. One is related to alkylating agent chemotherapy and characterized by a latency period of up to 10 years (median 4-5 years), a preleukemic phase myelodysplasia (MDS), myelocytic type and poor response to therapy (Le Beau *et al.*, 1986, Rubin *et al.*, 1991). The distinctive chromosomal abnormalities in sAML and sMDS after alkylating agent chemotherapy with or without radiation are preferentially unbalanced translocations. Deletions or loss of chromosomes 5 and/or 7 (73% of cases) were significantly associated with previous therapy with alkylating agents ($p=0.002$). Other chromosomal abnormalities seen in sAML after alkylating agent therapy were: deletions of various parts of the short arm of chromosomes 20 and 17, and of the long arm of chromosome 20; loss of a whole chromosome 18; gain of a whole chromosome 8 or of the long arm of chromosome 1 (Pedersen-Bjergaard *et al.*, 1994, Pedersen-Bjergaard *et al.*, 1995).

DNA topoisomerase inhibitors administered with or without radiation are associated with a second class of sAML with a shorter latency period (<3 years), short-term or no myelodysplasia, predominantly monocytic or myelomonocytic type and cytogenetic abnormalities which are mostly balanced translocations. Balanced translocations involving bands 11q23 (translocations t(4;11), t(6;11), t(9;11), t(11;19)), 21q22 (t(3;21), t(8;21)) and 3q26 (56% of cases all together) were significantly associated with previous

therapy with drugs targeting topoisomerase II ($p<0.00005$). Other balanced translocations such as inv(16), t(8;16), t(15;17) and t(6;9) were also seen in sAML following topoisomerase II inhibitor chemotherapy. As topoisomerase inhibitors in most regimens are administered in combination with either cisplatin and/or alkylating agent, the sAML or sMDS arising after the combination chemotherapy has the characteristics and chromosomal abnormalities related to the exposure to both the alkylating agents (61% of cases with -5/5q-, -7/7q- losses) and topoisomerase II inhibitors (30% of cases with balanced translocations to bands 3q26, 11q23 and 21q22) (Pedersen-Bjergaard *et al.*, 1995).

The etiology of sAML and sMDS is not understood, however, the particular characteristics and chromosomal abnormalities seen in two classes of sAML are similar to those found in *de novo* AML. Moreover, the role of chromosomal abnormalities in the evolution of AML has been investigated (Pedersen-Bjergaard *et al.*, 1994, Pedersen-Bjergaard *et al.*, 1995). It was noticed that *de novo* AML patients with unbalanced aberrations, in particular deletions of chromosomes 5 and 7, were often older and in many cases had a history of past exposure to mutagenic chemicals. On the other hand, *de novo* leukemias with balanced translocations, especially to chromosome band 11q23, predominated in neonatal leukemia, and the AML with balanced translocations were also observed more frequently in children and young adults. It was proposed that the increasing incidence with age of the *de novo* AML (or sAML) with unbalanced chromosome aberrations could be related to a life-long (or cancer therapy with alkylating agents in the case of sAML) exposure of the general population to irradiation and to the chemical carcinogens. While the less age-dependent development of *de novo* AML (or

sAML) with balanced chromosomal aberrations could point to alternative mechanism in leukemogenesis, such as spontaneous illegitimate recombination during the normal activity of DNA-topoisomerase II (or induced by topoisomerase II inhibitor chemotherapy in the case of sAML) (Pedersen-Bjergaard *et al.*, 1994).

The specificity of sAML in relation to the types of drugs used in chemotherapy suggests that the mutagenic and genotoxic effects of the drugs play a crucial role in the leukemogenic process. Etoposide (topoisomerase II inhibitor) and melphalan (alkylating agent) are among the most frequently used chemotherapy agents. Etoposide and melphalan are also unique in that each of them is used as a single agent in the treatment of groups of small cell lung cancer (SCLC) and multiple myeloma (MM) patients respectively. Because of their involvement in secondary leukemia and because of their wide use in chemotherapy, the ability of these drugs to cause long-lasting genetic damage has been extensively studied. Great progress has been made in our understanding of the mechanisms of action of these drugs, as well as of their mutagenicity and carcinogenicity.

2. Genetic profile of chemotherapy agent etoposide.

2.1 Chemical structure, history and current use.

The anticancer drug etoposide (VP16-213, VP-16 or 4'-demethylepipodophyllotoxin 9-[4,6-0(R)-ethylidene-β-D-glucopyranoside, $C_{29}H_{32}O_{13}$) is a semisynthetic derivative of podophyllotoxin (Figure 1.1). The podophyllotoxins have been used therapeutically for over 1000 years. A medieval textbook of the 10th century AD, "The Leech Book of Bald", describes the treatment of cancer with extracts from roots of wild chervil known to contain deoxypodophyllotoxins (Cockayne, 1961).

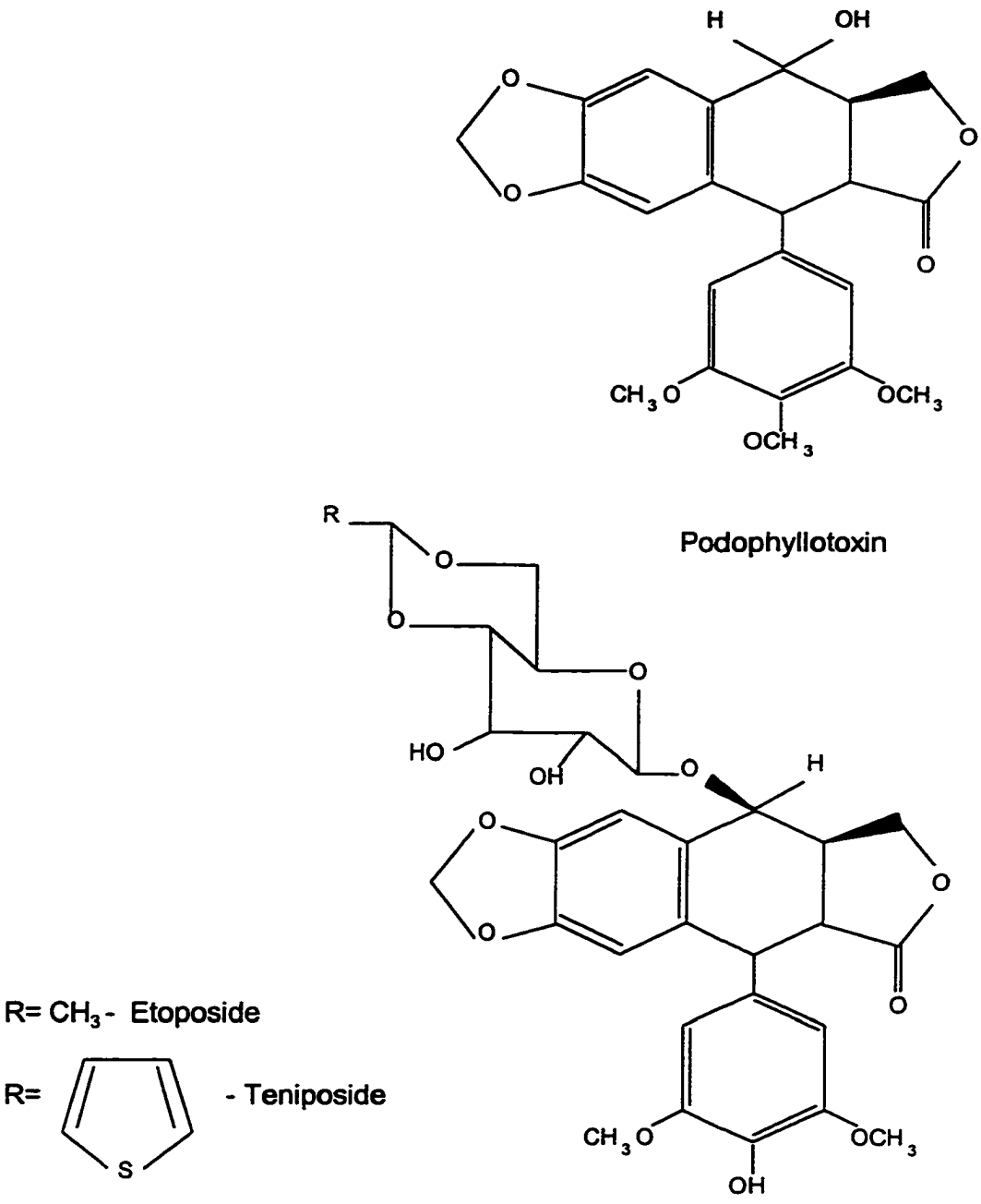


Figure 1.1 Chemical structures of podophyllotoxin and its derivatives etoposide and teniposide.

The medicinal properties of extracts from *Podophyllum peltatum* (mayapple or American mandrake) or *Podophyllum emodi* have been recognized by American Indians and inhabitants of the Himalayas respectively for more than two centuries (Lombard, 1954). In the 19th century, the resin podophyllin was extracted and recommended for diseases of the liver and kidneys, scrofula, syphilis, gonorrhoea, obstructed menstruation and coughs (Kelly *et al.*, 1954). In the 1860s podophyllin was found to be topically effective for skin cancers (Clark *et al.*, 1987). The original podophyllotoxin, a podophyllin compound, was evaluated as an antineoplastic agent in the 1950s. Although clinical activity was demonstrated, its clinical use was limited due to severe gastrointestinal toxicity (Clark *et al.*, 1987).

In the 1960s several podophyllotoxin derivatives, including the glycosides etoposide and teniposide, were developed by Sandoz Pharmaceuticals Corporation (Hanover, New Jersey) in attempts to synthesize compounds with better therapeutic index (Hande, 1992). The biological activities of the various compounds were examined against mouse P-185 mastocytoma cells and in the mouse L1210 leukemia system. Based on these studies etoposide and teniposide, that showed the highest activity, were selected for clinical trials (Stahelin, 1973). Etoposide was introduced into clinical trials in 1971 and approved by the Food and Drug Administration for marketing under the trade name VePesid (Bristol-Myers Squibb Company, Evansville, Indiana) in early 1984 (Hande, 1992). Both vials for intravenous injection and oral capsules (approved in 1987) of etoposide became available for use in cancer chemotherapy.

Etoposide as a single agent or in combination with other drugs demonstrated activity against a wide variety of cancers and it is currently used as a first-line drug in the

treatment of SCLC, germ cell tumors and lymphomas (Clark *et al.*, 1987, Yarbro (ed), 1992). Etoposide also exhibits single agent activity in acute leukemias. Other tumors in which etoposide is active include Kaposi's sarcoma, gestational trophoblastic choriocarcinoma and ovarian carcinoma.

2.2 Intracellular target.

In contrast to their parental compound, podophyllotoxin, which shows potent antimitotic activity through tubulin binding and cell arrest in mitosis, neither etoposide nor teniposide produce any effect on tubulin assembly (Loike *et al.* (1), 1976). Teniposide and etoposide induce a premitotic block in the cell-cycle and are most cytotoxic in the late S or early G2 phases *in vitro* and *in vivo* (Clark *et al.*, 1987). These are, therefore, phase-specific cytotoxic drugs. Treatment of cells with etoposide and related compounds reduces nuclear DNA to a lower molecular weight form in a dose- and temperature-dependent manner which is reversed upon the drug removal (Loike *et al.* (2), 1976). This effect is not observed when the drug is incubated with isolated purified calf thymus DNA, indicating that DNA breakage requires some intracellular component (Loike *et al.* (2), 1976). Using the alkaline elution technique, single- and double-strand DNA breaks as well as DNA-protein crosslinks are observed following etoposide treatment (Wozniak *et al.*, 1983). The DNA breakage is also observed in a temperature-dependent manner when isolated nuclei are exposed to the drug, the process dependent on magnesium and stimulated by the presence of ATP (Glisson *et al.*, 1984). The drug effect was lost when isolated nuclei were extracted with 0.35 NaCl and was reconstituted when the protein extract was added back to the isolated nuclei. Minocha and Long (1984) observed inhibition of DNA catenation or strand passage activity by DNA topoisomerase II

enzyme *in vitro* in the presence of low doses of etoposide and related compounds and proposed the role of this enzyme in the DNA breakage activity of these drugs. That was confirmed by the fact that the structure activity relationships for enzyme inhibition paralleled those of *in vivo* DNA breakage and cytotoxicity (Long *et al.*, 1984). The hypothesis that topoisomerase II was responsible for the degradation of nuclear DNA by etoposide and teniposide was strengthened by the studies of monkey cells infected with SV40 virus. These studies showed that the drug-induced cleavage sites on intracellular viral DNA were similar to those obtained when purified viral DNA was incubated with calf thymus topoisomerase II in the presence of these compounds (Yang *et al.*, 1985 (1), Yang *et al.*, 1985 (2)). The studies also shown that intracellular viral DNA became protein linked following drug exposure and that this complex could be immunoprecipitated by antisera against topoisomerase II (Yang *et al.*, 1985 (2)). Thus, evidence was obtained that epipodophyllotoxin derivatives etoposide and teniposide interact with a nuclear enzyme topoisomerase II in a way that makes it incapable of strand passage activity and which, in the presence of denaturing agents, is seen as protein associated single and double-strand DNA breaks.

DNA topoisomerases are enzymes that control and modify the topological states of DNA (reviewed in Wang 1985). These enzymes can catalyze many types of interconversions between DNA topological isomers (topoisomers) such as relaxation of positively or negatively supercoiled DNA, knotting/unknotting and catenation /decatenation. These reactions are vital in such metabolic cellular processes as transcription, replication, chromatid and chromosome segregation, chromosome condensation/decondensation and recombination. Topoisomerases have been found in

prokaryotes (DNA gyrase analogous to type II enzyme and DNA topoisomerase III similar to type I enzyme in *E.coli*) and in eukaryotes.

There are two types of topoisomerases in eukaryotes. Type I topoisomerase transiently breaks a DNA strand and allows the other strand to pass through the transient break, while type II topoisomerase transiently breaks a pair of complementary strands allowing the passage of another double-stranded DNA (reviewed in Liu 1989). Contrary to topoisomerase I, topoisomerase II is a homodimer and requires divalent cations and hydrolysis of two ATP molecules for its action. Human DNA topoisomerase II is a homodimer encoded by a single-copy gene on chromosome band 17q21-22. Dimer subunits each contain at least two separate domains. One domain is likely used to mediate ATP hydrolysis and the other domain is involved in DNA breakage. While topoisomerase I is primarily involved in transcription, topoisomerase II appears to be more important in maintaining chromosomal structure and organization. Topoisomerase II is a component of the interphase nuclear matrix and mitotic chromosome scaffold (Figure 1.6). Genomic DNA associates with these structures to form topologically constrained loops of DNA, five to several hundred kilobases in length (Figure 1.6). Genomic DNA sequences that interact with nuclear matrix or mitotic scaffold are referred to as matrix associated regions (MARs) or scaffold associated regions (SARs) respectively (Blasquez *et al.*, 1989). Topo II binding and teniposide induced DNA cleavage by topoisomerase II have been demonstrated to occur within these nuclear matrix associated regions (Razin *et al.*, 1991).

The mechanism of topoisomerase action is similar for both topoisomerase types (reviewed in Anderson *et al.*, 1994). Type II topoisomerase binds noncovalently to

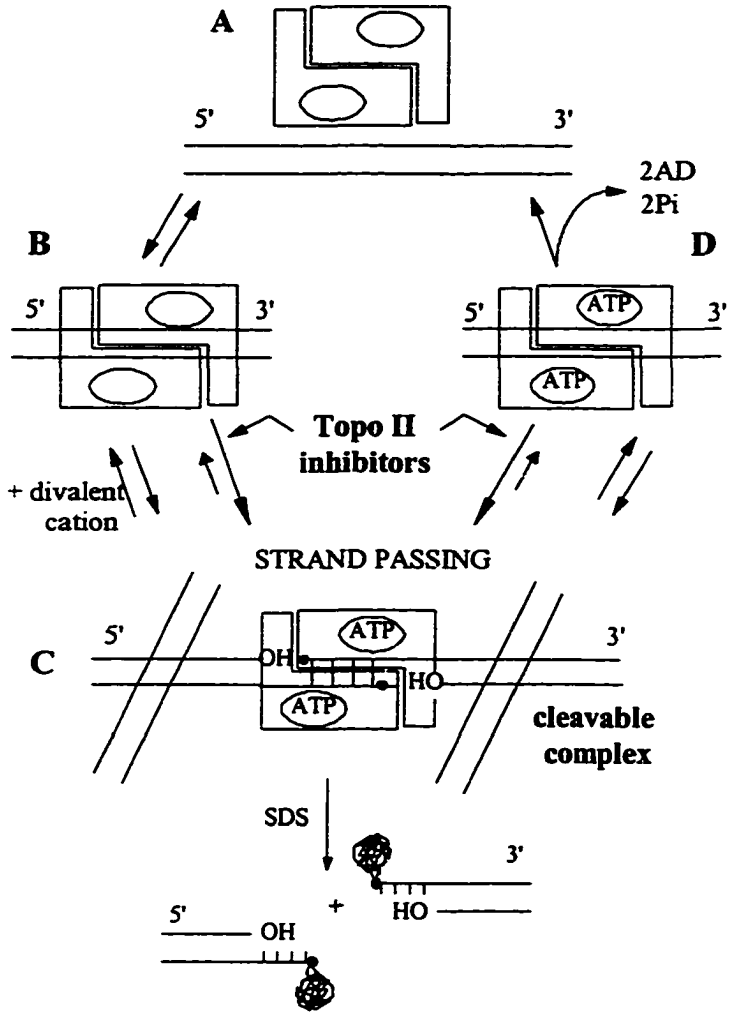


Figure 1.2 Action of topoisomerase II and its inhibitors. (A) Topoisomerase II forms a homotypic dimer. Dimer subunits each contain at least two separate domains, one of which is likely to mediate ATP hydrolysis and another mediates DNA breakage. (B) The enzyme binds noncovalently to DNA in the absence of either divalent cations or ATP. The formation of topoisomerase II-DNA complexes is enhanced by ATP. (C) Addition of cations leads to double-stranded DNA cleavage and concomitant covalent binding of one dimer subunit to each of the complementary DNA strands. Formation of this complex is reversible and exists in equilibrium with strand religation. Topoisomerase II-mediated "cleavable complexes", presumed to be reaction intermediates, are recovered by treating reaction mixtures with protein denaturants (SDS). Topoisomerase II-DNA complexes contain enzyme subunits with phosphotyrosyl linkages to the 5'-ends of breaks in both strands of the DNA duplex. Breaks on opposing strands are usually separated by 4 bases, thus forming a staggered double-strand break. Breaks occur nonrandomly at discrete sequences within a given DNA molecule. Many agents (topoisomerase II inhibitors) enhance the recovery of these complexes because they inhibit the religation reaction. When ATP binds to the enzyme, the reactions catalyzed by topoisomerase II are completed via passage of a duplex DNA strand through the double-strand break covalently bound to enzyme subunits. (D) The enzyme may remain bound to DNA with strand breaks existing in equilibrium between cleavage and religation until ATP hydrolysis occurs. Enzyme turnover requires the net hydrolysis of two ATP molecules. (Adapted from Anderson *et al.*, 1994).

DNA in the absence of either divalent cations or ATP (Figure 1.2). ATP enhances the formation of enzyme-DNA complexes. Addition of cations leads to double-stranded DNA cleavage and concomitant covalent binding of one dimer subunit to each of the complementary DNA strands. Formation of this enzyme-DNA cleavable complex is reversible and exists in equilibrium with strand religation. Cleavable complexes contain enzyme subunits with phosphotyrosyl linkages to the 5'-ends of breaks on both strands of the DNA duplex. Topoisomerase II- induced strand breaks on opposing strands are usually separated by 4 base pairs (Figure 1.2). The reaction catalyzed by the topoisomerase II is completed via passage of a duplex DNA through the double-strand break. The enzyme remains bound to DNA with strand breaks existing in equilibrium between cleavage and religation until ATP hydrolysis occurs. Enzyme turnover requires the net hydrolysis of two ATP molecules. Opposition of the double-strand ends throughout the reaction is maintained by self-adhesion of the topoisomerase II subunits. A protein clamp model has been proposed for eukaryotic DNA topoisomerase II (Figure 1.3) (Roca *et al.*, 1992). In this model a homodimeric enzyme acts as an ATP-modulated protein clamp: ATP binding closes the two jaws of the clamp, and hydrolysis of the bound ATP precedes the reopening of the clamp (Figure 1.4). As a DNA-bound protein clamp closes its jaws, it can capture a second DNA segment and transport it through an enzyme-mediated gate in the bound DNA.

Variations in the intracellular levels of topoisomerases are consistent with their function. Two forms of topoisomerase II have been identified in mammalian cells, α and β, which have slightly different molecular weights and are encoded by separate

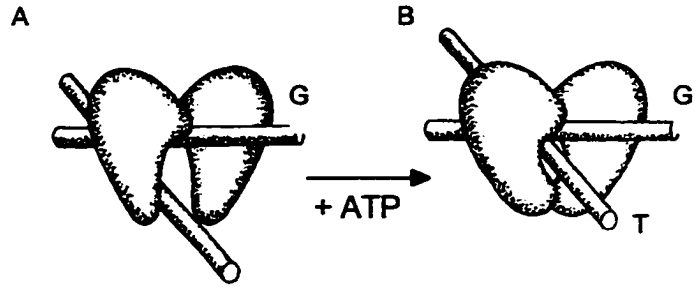


Figure 1.3 An illustration of a topoisomerase II as an ATP-dependent protein clamp. (A) In the absence of ATP, the clamp is open whether the enzyme is by itself or is bound to a DNA segment (G segment). In this open state, a second DNA segment (T) can enter the molecular trap . (B) The binding of topoisomerase II closes the clamp. If a T segment is present in the trap at the time of closure, it is caught for transportation through the enzyme-mediated DNA gate in the G segment. (Adapted from Roca *et al.*, 1992.)

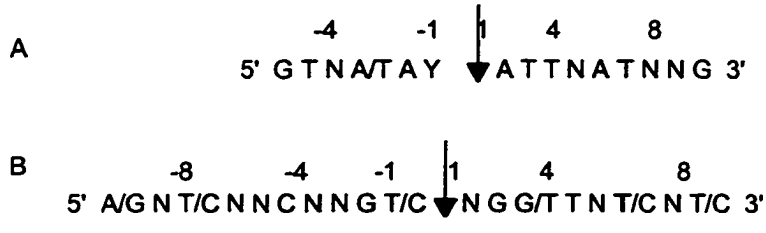


Figure 1.4 Consensus DNA binding and cleavage sites for *Drosophila* (A) and vertebrate (B) topoisomerase II. N is any base and cleavage occurs at the arrow between -1 and 1 bases.

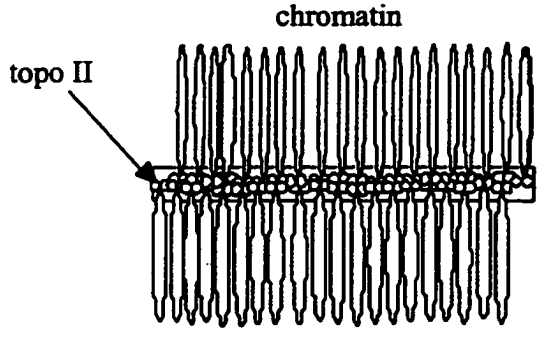


Figure 1.6 Topoisomerase II and its relationship to the chromatin. The enzymes are at the base of the chromatin loops and appear to anchor matrix association regions (MARs) to the nuclear matrix or mitotic scaffold. (Adapted from Ferguson *et al.*, 1996.)

genes (Hsieh 1992). Topoisomerase II α level is elevated in rapidly proliferating cells: it increases during S phases, reaches peak at G_2/M phases of the cell cycle and generally decreases in differentiated cells. Topoisomerase II β, on the other hand, exists at a constant level throughout the cell cycle and is present at a lower level in some differentiated cell types. These findings suggest that the primary role of topoisomerase II α is to resolve conformational constrains during replication and topoisomerase II β plays a role in gene regulation and chromosomal organization. Topoisomerase I expression does not alter throughout the cell cycle indicating its involvement in constantly occurring processes such as transcription (Hsiang *et al.*, 1988).

2.3 Mechanism of topoisomerase inhibitors action.

A variety of agents interfere with the action of topoisomerases such as nalidixic acid, a DNA gyrase inhibitor, camptothecin, a topoisomerase I inhibitor, and series of compounds such as acridines, anthracyclines, epipodophyllotoxins and coumarins, inhibitors of topoisomerase II (Anderson *et al.*, 1994). These agents have various characteristics, but the most important common feature shared by almost all of them is that they inhibit the topoisomerase action by stabilizing the cleavable complex involving enzyme, agent and DNA. The agents shift the equilibrium to stabilize the covalently bound form of topoisomerase. The frequency of cleavable complexes recovered with protein denaturants as protein-bound DNA strand breaks is greatly enhanced by topoisomerase inhibitors. At relatively low concentrations, most of these agents enhance cleavable complex recovery before inhibition of overall enzyme catalytic function. Removal of agent or addition of salt to dissociate non-covalently bound protein reduces

the recovery of agent potentiated protein cross-linked DNA strand breaks which is consistent with a reversible drug effect (Liu, 1989). Incomplete resolution of breaks occurs with some DNA-intercalating agents which may break DNA by additional mechanisms that do not involve topoisomerase (Liu, 1989) and a sub-population of irreversible enzyme-linked strand breaks can by induced by topoisomerase I inhibitors (Champoux, 1990). Studies with both topoisomerase I and II suggest that agents which stabilize the cleavable complexes do so by inhibiting the religation reaction catalyzed by the enzyme (Champoux, 1990, Robinson *et al.*, 1991).

Topoisomerase-active agents usually produce more single-strand than double-strand DNA breaks. For, example, etoposide produces a ratio of single-strand/double-strand DNA breaks of 5-6:1 (Spiridonitis *et al.*, 1989). This ratio increases with increasing drug concentration consistent with double-strand break mechanism of topoisomerase II (Long *et al.*, 1985). Concentration-dependent increase in double-strand breaks correlates with other genotoxic events such as SCE and chromosomal aberrations (Pommier *et al.*, 1985, Pommier *et al.*, 1988). Induction of strand breaks correlates with cellular levels of topoisomerase II (Sullivan *et al.*, 1987) and is reduced in cells with mutant forms of topoisomerase II (Liu 1989).

Many of topoisomerase-interactive agents are also capable of inhibiting the catalytic properties of enzymes but at concentrations higher than those which stabilize cleavable complexes (Tewey *et al.*, 1984). This property is probably more potent in those topoisomerase inhibitors that are DNA intercalators. DNA-intercalating agents are planar aromatic molecules which can insert between adjacent base pairs stacked in the DNA helix which causes DNA unwinding and other DNA distortions (Wilson *et al.*, 1981).

This distortion may prevent topoisomerases from forming the intermediate cleavable complexes. In fact, at high concentrations at which topoisomerase-II mediated strand passage is inhibited, some intercalating agents inhibit their own ability to stabilize cleavable complexes in addition to inhibiting the formation of cleavable complexes induced by other topoisomerase-II inhibitors (Zwelling *et al.*, 1991). Etoposide is a non-intercalating and non-binding to DNA topoisomerase II inhibitor.

2.4 Sequence specificity.

There is a high degree of specificity in DNA recognition sequences by topoisomerases. Consensus DNA binding sequences have been determined for both types of topoisomerases (Champoux 1990, Sander *et al.*, 1985, Spitzner *et al.*, 1988). The consensus sequences of 15 and 18 base pairs long have been determined for the *Drosophila* and chicken topoisomerase II enzymes respectively (Figure 1.4) (Sander *et al.*, 1985, Spitzner *et al.*, 1988). Topoisomerase II from human placenta was shown to cleave DNA at the sites essentially identical to those cleaved by the chicken enzyme, suggesting that vertebrate type II enzymes share a common catalytic sequence. The consensus accurately predicts topoisomerase sites *in vitro*. A search for 80% matches to the consensus sequence will detect stretches of alternating purine-pyrimidine sequence and it was demonstrated that topoisomerase II is acutely reactive towards sequences that contain poly purine/pyrimidine regions (Spitzner *et al.*, 1988).

A 16-base pair double-stranded region was established as the minimal duplex region located symmetrically around the 4-base staggered cleavage site required for the topoisomerase II cleavage activity (Lund *et al.*, 1990). Topoisomerase II-mediated cleavage within the 16-base pair core duplex, however, required single-stranded regions

flanking the duplex at either side, or an extension at both ends of the duplex with 1 or more base pairs. The frequency of cleavable complex formation varied at separate sites and the complexes could occur at sites that do not conform to the consensus. Therefore, DNA sequence surrounding the consensus strongly influences the frequency of cleavable complex formation *in vitro*.

Topoisomerase inhibitors selectively enhance the recovery of topoisomerase cleavable complexes to varying degrees at specific sites (Spitzner *et al.*, 1988). While consensus was derived using the *m*-AMSA, an intercalating topoisomerase II inhibitor, it was equally valid for predicting topoisomerase II binding and cleavage without drugs (Spinzner *et al.*, 1988). In the presence of drugs new cleavage sites were detected that were not detected in their absence (Spitzner *et al.*, 1988). Cleavage enhancement by teniposide and *m*-AMSA was shown to have strong dependence on bases located directly at the sites of cleavage (Pommier *et al.*, 1991). The preferred bases are C at the 3' terminus for teniposide and A at the 5' terminus for *m*-AMSA. For doxorubicin, cleavage requires the presence of A at the 3' terminus of at least one of the pair of breaks at the double-strand cleavage. It was proposed that topoisomerase inhibitors do not necessarily bind the enzyme but, rather, may stack with one or the other base pair flanking the enzyme cleavage sites (Pommier *et al.*, 1991).

2.5 Mechanism of cytotoxicity.

The mechanism of etoposide cytotoxicity is not completely understood. It is believed that DNA strand breakage is the initial event in the sequence of changes leading to cytotoxicity. A close relationship between DNA scission and cytotoxicity has been demonstrated (Wozniak *et al.*, 1983). Additionally, drug resistant cell lines had little

DNA strand breakage even at a very high drug concentration (Spiridonitis *et al.*, 1989 Chatterjee *et al.*, 1990). Teniposide has been shown to be 5-10 times more potent than etoposide in the formation of strand breaks and cytotoxicity (Long *et al.*, 1985). However, a rapid exponential repair of both single and double strand breaks after the drug withdrawal from the medium suggests that there are processes other than DNA breaks that lead to cell death (Long *et al.*, 1985). Chatterjee *et al.* (1990) studied cell lines with reduced activity of poly(ADP-ribose) polymerase and found them to be resistant to etoposide-induced cytotoxicity, however, etoposide-induced formation of protein-cross-linked DNA strand breaks and their repair after drug removal was not affected in all cell lines. These studies showed a clear dissociation between induction of DNA strand breaks and cytotoxicity. On the other hand, a good correlation was observed between drug-induced SCE and cytotoxicity. Moreover, time-course of etoposide-induced cytotoxicity correlated better with the time course of SCE induction rather than with the protein-cross-linked DNA strand break formation (Chatterjee *et al.*, 1990). A defect in the ability to mediate SCE was proposed as a mechanism of drug resistance in these poly(ADP-ribose) synthesis-deficient cell lines (Chatterjee *et al.*, 1990). Thus, it was proposed that the drug-induced stabilization of topoisomerase II-DNA complexes stimulated the induction of SCE that consequently leads to cell death.

The dilemma between etoposide-induced cytotoxicity and topoisomerase II-DNA complexes is further illustrated by the demonstration of the cell cycle- dependent dissociation between drug-induced protein crosslinked DNA strand breaks and cytotoxicity. Cells were shown to undergo maximum etoposide-induced strand breaks during G2/M phase when the total topoisomerase II levels peak, while exposure during S

phase caused maximum cytotoxicity (Chow *et al.*, 1987). This data suggest that the important cytotoxic mechanisms of etoposide and other Topo II-interactive agents involve processes associated with DNA replication. Further, DNA replication inhibitors protected cells against the cytotoxicity of etoposide but did not affect the formation of cleavable complexes or protein-linked DNA strand breaks suggesting that the blockage of replication fork progression may signal cell death (Holm *et al.*, 1989). Taken together these findings prompted the hypothesis that etoposide exposure leads to the stabilization of the Topo-II-DNA complexes, which block the replication fork progress, and consequently to the activation of a replication block bypass mechanism via strand exchange leading to SCE (Berger et al., 1991). The alternative proposed mechanism of SCE induction by Topo II inhibitors is subunit exchange model. According to this model drug-stabilized cleavable complexes on adjacent newly synthesized portions if sister chromatids exchange homologous Topo II subunits and in doing so, exchange the DNA strands to which they are covalently bound (Anderson *et al.*, 1994). Non-homologous recombination resulting from SCEs in turn leads to genomic deletions or insertions that may inactivate essential gene products and eventually lead to cell death (Figure 1.5).

This hypothesis explained the dissociation between the cleavable complex formation and cytotoxicity. It also suggests that cytotoxicity parallel the induction of SCE and mutation. Various studies of etoposide cytotoxicity confirmed the proposed hypothesis. Thus, Singh and Gupta (1983) showed that the frequency of etoposide-induced SCE correlated with the rate of mutation induction at the *hprt* gene in CHO cells. The majority of etoposide induced mutations at this locus in V79 cells were partial deletions and /or rearrangements (Berger *et al.*, 1991). At the same time, 11% of teniposide-induced

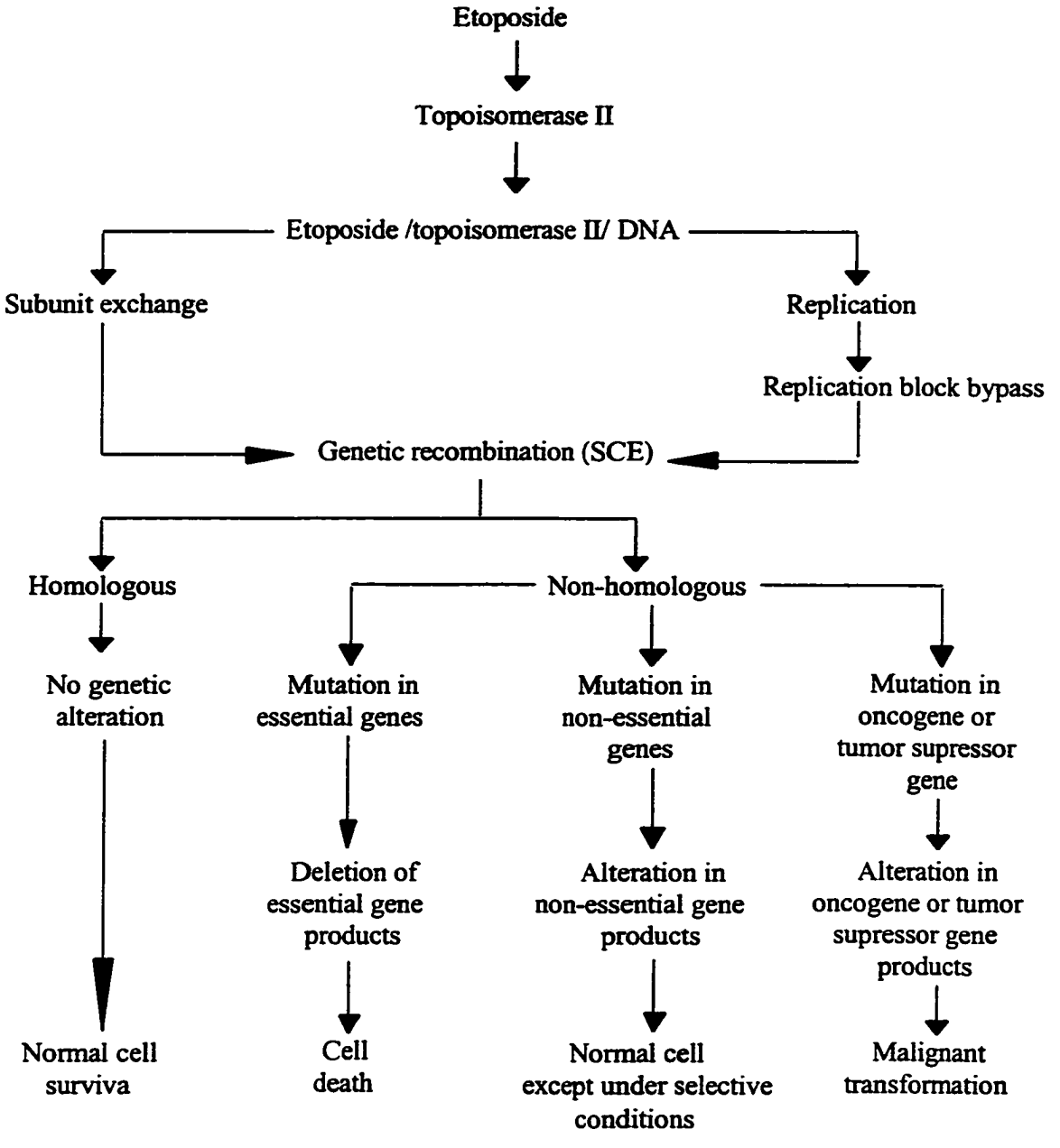


Figure 1.5 Proposed pathway for etoposide-induced cytotoxicity and genetic alterations. (Adapted from Hashimoto *et al.*, 1995.)

mutants at the *aprt* gene in CHO D422 cells were missing a cDNA-PCR product compared to the absence of such events in the control spectrum (Han *et al.*, 1993). While these events were measured in the nonessential genes, it is likely, that the same events occur in essential genes as well. Another support for the proposed mechanism of etoposide cytotoxicity comes from the fact that DNA crosslinks (drug-stabilized cleavable complexes in the case of topoisomerase II inhibitors) are well established as stimuli for SCE formation during DNA replication (Painter, 1980).

The proposed mechanism for etoposide cytotoxicity explains the requirement for DNA replication to foster etoposide-induced SCE formation and cytotoxicity (Dillehay *et al.*, 1987). It may also explain why cells are most sensitive to killing by etoposide during S phase, when SCE takes place, and why they die after short exposure to topoisomerase II-interactive agents despite the rapid resolution of protein-linked strand breaks. Cells may become "programmed for death" by non-homologous SCE during the period of drug exposure.

Like other DNA damaging agents, etoposide was shown to induce apoptosis in a variety of systems (Kaufman *et al.*, 1989). Studies with apoptosis inhibitors provided further support for the described above mechanism of etoposide cytotoxicity. An apoptosis inhibitor IL-3 was shown to inhibit the cytotoxic effects of etoposide, although the extent of DNA strand breaks and their repair were not affected by IL-3 (Collins *et al.*, 1992). Kamesaki *et al.* (1993) have shown that overexpression of human bcl-2 protein in CH31 mouse cells inhibited the etoposide-induced apoptosis and cytotoxicity but had little or no effect on the production of DNA-protein cross-links or DNA single- and double-strand breaks. Later Hashimoto *et al.* (1995) have shown that overexpression of

human bcl-2 protein in CHO cells was associated with a reduction in etoposide-induced genetic recombination measured by SCE production. At the same time there was a reduction in mutation as measured at the *hprt* gene and cytotoxicity based on colony formation, while the overexpression did not affect the protein cross-linked DNA single-strand breaks. Therefore, the cytotoxicity of etoposide could be dissected into early events (DNA-strand breaks mediated by topoisomerase II) and later events (secondary DNA fragmentation and cell death). IL-3 and bcl-2 inhibit apoptosis and cytotoxicity at some steps between these two events. It is still unknown, however, by which mechanism the initial DNA damage induces apoptosis. The induction of p53-dependent apoptosis pathway following DNA damage has been proposed (Kamesaki *et al.*, 1993).

To summarize the current model for the etoposide and other topoisomerase II inhibitors cytotoxicity, exposure to increasing drug concentrations would lead to an increase in both homologous and nonhomologous recombination (Anderson *et al.*, 1994) (Figure 1.5). Homologous recombination creates no genetic change and will not contribute to the cytotoxicity. Nonhomologous recombination, on the other hand, causes gene deletions and recombination, the consequences of which will depend on the gene affected. If the essential gene is affected its deletion should lead to a loss of essential gene product and cell death (likely through the apoptosis pathway). If a nonessential gene is affected it should not affect the survival but will increase the frequency of mutation in that gene (like *hprt* and *aprt*). Alteration of an oncogene or a tumor suppressor gene on the other hand will lead to malignant transformation (like in the cases of sAML). This hypothesis provides a unifying mechanism of how etoposide can induce cytotoxicity and

increase mutation frequency as well as induce secondary leukemia with characteristic chromosomal abnormalities.

While apoptosis is a common pathway of cell death after etoposide exposure, there is heterogeneity of cell death mechanisms after exposure to topoisomerase II inhibitors among human cancer cell lines. Thus, while both cell lines HT-29 and HL-60 showed similar loss of colony formation ability after teniposide treatment, secondary DNA fragmentation indicative of apoptosis was detected in HL-60 but not in HT-29 cells (Bertrand *et al.*, 1991). It was proposed that *c-myc* overexpression in HL-60 cells but not in HT-29 may be involved in apoptosis.

An alternative mode of death, mitotic catastrophe or reproductive death, has been observed in human tumor cell population. *bcl-2* overexpression in HeLa S3 cells rendered cells resistant to apoptosis-inducing effects by etoposide but had no significant effect on clonogenic survival of HeLa cells exposed to etoposide due to appearance of giant, multinucleated cells incapable of reproduction (Lock *et al.*, 1996).

Cytotoxicity of etoposide is enhanced by caffeine, an inhibitor of DNA repair pathways (Clark *et al.*, 1987). However, caffeine was also shown to attenuate the action of etoposide (Perez *et al.*, 1994 (2)). The presence of 5-10 mM caffeine greatly reduced the growth inhibition, G2-arrest and increase in cell size after pulse etoposide treatment of U-937 cells possibly by inhibition of RNA synthesis. Transcription inhibitors were shown to reduce the etoposide-induced growth inhibition in a similar manner. One possible explanation is that interaction between the transcription machinery and topoisomerase II-cleavable complexes leads to the formation of irreversible double-strand breaks that may later arrest replication forks as cells enter S-phase. Caffeine also partially

prevented the etoposide-induced increase in the number of topoisomerase II-DNA cleavable complexes (Perez *et al.*, 1994 (2)). Lock *et al.* (1994) found that the action of caffeine may depend on concentration and time of exposure. When applied for 24h following etoposide exposure in HeLa cells, caffeine enhanced cytotoxicity in a concentration-dependent manner by alleviating G2 arrest and increasing the incidence of mitotic death. Brief etoposide exposures (5-10 mM for 1-2 h) caused mitotic progressions to a limited extend in cells that were arrested in G2 following etoposide treatment. However, longer exposure times at 10 mM caffeine caused inhibition of both cell cycle progression and mitotic death, and the enhancement of etoposide cytotoxicity could be accounted for by up to 3-fold increase in the proportion of apoptotic cells. Thus, caffeine potentiates etoposide cytotoxicity by two morhpologically distinct mechanisms depending on its concentration (Lock *et al.*, 1994).

While DNA replication was established to be necessary for the cytotoxic effect of etoposide (see above), the etoposide cytotoxicity was only partially blocked by DNA polymerase inhibitor, aphidicolin, presumably because etoposide has both S-phase specific and cell cycle non-specific mechanisms of cytotoxicity (Holm *et al.*, 1989).

2.6 Cell cycle arrest.

Exposure of proliferating cells to DNA-damaging agents generally results in either G1 arrest which allows excision repair mechanism to remove DNA adducts before DNA synthesis or a G2 arrest where post-replication repair mechanisms attempt to repair DNA damage before cell division (Afshari *et al.*, 1993). The G2 arrest is an important cell-cycle checkpoint at which the signal for either mitosis or apoptosis is given, most probably at the level of the p34cdc2-cyclin B complex (Shi *et al.*, 1994).

Etoposide has been shown to arrest cells in G2 phase of the cell cycle through the inhibition of p34cdc2 kinase activity (Lock *et al.*, 1990) and inhibit S phase cycle traverse (Krishan *et al.*, 1975) consistent with its mechanism of action and cytotoxicity.

2.7 Alternative mechanism of action.

An alternative mechanism of etoposide cytotoxicity has been proposed whereby etoposide is activated by a dehydrogenase that converts it's 4' phenolic group to a semiquinone free radical (Wozniak *et al.*, 1984). This mechanism is suggested from the results where selected substrates and inhibitors of dehydrogenases and free radical scavengers inhibited the DNA breakage potential by etoposide and cytotoxicity (Wozniak *et al.*, 1984). Several enzymatic systems metabolize etoposide to products capable of irreversible binding to proteins and DNA (Clark *et al.*, 1987). *In vitro* evidence has demonstrated that the quinone derivatives of etoposide and teniposide can produce DNA damage and cytotoxicity (Clark *et al.*, 1987).

2.8 Apoptosis.

Etoposide was shown to induce apoptosis in a variety of cell types. As a DNA damaging agent and inducer of DNA strand breaks, etoposide is expected to induce apoptosis through the p53-dependent pathway. However, results show that there are many mechanisms by which the topoisomerase II inhibitors may trigger apoptosis.

Clarke *et al.* (1993) showed that p53 status exerts a significant and dose-dependent effect in the initiation of apoptosis in thymocytes. Thymocytes with homozygous null and heterozygous for p53 deletion were resistant or partially resistant to apoptosis respectively but only when it was induced by etoposide and other agents that cause DNA-strand breakage (Clarke *et al.*, 1993). Etoposide has been shown to induce apoptosis in

thymocytes and mouse fibroblasts dependent on new protein and RNA, but not DNA synthesis (Walker *et al.*, 1991, Mizumoto *et al.*, 1994). In rat thymocytes apoptosis was induced in G0/G1, G2/M and S phases of the cell cycle (Fearnhead *et al.*, 1994).

Interestingly, topoisomerase II inhibitors that do not stabilize a DNA cleavable complex, were shown to induce thymocyte apoptosis, suggesting that direct DNA cleavage is not essential for induction of apoptosis by topoisomerase II inhibitors (Onishi *et al.*, 1994). These authors suggested that the alteration of DNA helicity induced by a subtle inhibition of topo II activity might have an important role in the induction of apoptosis in thymocytes.

Two types of apoptosis were observed in human B cells and murine T and B cells after etoposide exposure one of which was associated with small DNA fragmentation (180-200 bp) or a typical "ladder" and the other had large DNA fragments (100-150kbp) (Fournel *et al.*, 1995). Thus, the type of apoptosis appears to be an intrinsic property of each cell type.

While in the majority of cells the absence of p53 expression leads to a dramatic increase of cellular resistance to DNA damaging agents including etoposide, this is not the case for HL-60 cells which have an extensive p53 deletion. HL-60 cells have no p53 expression but are highly sensitive to rapid apoptosis induced by etoposide (Solary *et al.*, 1994). This apoptosis was not prevented and even induced by protein synthesis inhibitor cycloheximide and RNA synthesis inhibitor actinomycin D. It was proposed that HL-60 cells constitutively express the apoptotic machinery that may be kept under control of a yet unknown repressor. Interestingly, relatively high levels of *bcl-2* expression in HL-60 cells were not sufficient to protect cells against apoptosis as it does in other cells (see

above). Therefore, there exists an alternative suicide pathway in HL-60 cells to allow for the commitment to cell cycle arrest and death.

2.9 Resistance.

Resistance to etoposide and other topoisomerase II-inhibitors is multifactorial. The mechanisms of resistance include: (a) amino acid substitutions in the topoisomerase II protein resulting in enzymes that are unresponsive to the drugs, (b) altered subcellular distribution of topoisomerase II isoforms, (c) decreased intracellular content of topoisomerase II, (d) reduced delivery of the inhibitor to the target enzyme via overexpression of membrane efflux pump (Sullivan *et al.*, 1991). Cells can also be rendered resistance to etoposide if they have resistance to apoptosis. Thus, human monocytic leukemia U937 cells treated with etoposide undergo apoptosis in the majority of cells in S phase within 2 h of the end of treatment. However, a mutant was isolated that was resistant to apoptosis and showed significant actual drug resistance. Both wild type and mutant cells, however, had the same levels of protein-DNA covalent links and DNA double-strand breaks caused by etoposide, indicating that the initial DNA damage was comparable, whereas the following cellular responses that resulted in apoptosis differed between the cell lines (Kataoka *et al.*, 1994).

There are many mechanisms by which cells can become resistant to apoptosis, as apoptosis is a complex process involving many genes and pathways. Some of the cellular factors leading to apoptosis and drug resistance include differential oncogene expression (*bcl-2*, *c-myc*, *c-jun* and *c-fos*) (Eliot *et al.*, 1995), action of growth factors (IL-3 and IL-6) (Borsellino *et al.*, 1995), *p53* gene mutation (Fan *et al.*, 1994) and cell cycle regulation or the ability of cells to arrest cell cycle in G2 or S phase (Dubrez *et al.*, 1995).

Matsumoto *et al.* (1997) studied the evolution of etoposide resistance and found that more than one mechanism can contribute to the resistant phenotype when increasing selective pressure is applied. Reduced expression of topoisomerase II is sufficient to confer substantial resistance early in the selection process with synergy from MDR-1 overexpression helping to confer high levels of resistance.

Sinha *et al.* (1991) have shown that the mechanism of resistance involves the absence of some cytosolic factors necessary for the maximum DNA cleavage or the presence of detoxifying enzymes in the resistant HL-60 cells compared to drug-sensitive cells.

2.10 Pharmacology and pharmacokinetics.

Bioavailability (fraction of administered drug that reaches systemic circulation) of oral etoposide (100 mg dose) is around 50%, however, a great interpatient variability exists with some patient absorbing 25 % of an administered dose while others absorb as much as 76% (Hande *et al.*, 1993). Mean peak plasma concentration of 3±1.8 μg/ml is achieved within 0.5-4 h (Hande *et al.*, 1993). The terminal elimination half-life is 5 to 10 h. Approximately 6 to 25% of unchanged etoposide is recovered in the urine and most of this excretion occurs in the first 12 h (Clark *et al.*, 1987). Etoposide is extensively bound to plasma proteins. Etoposide protein binding in cancer patients is variable and is related to the serum albumin and total bilirubin levels (Stewart *et al.*, 1990). The mean measured unbound percentage of etoposide in plasma is 15.3±11.6%. Patients with low serum albumin levels have greater toxicity from a given drug dose than patients with normal serum albumin concentrations. Renal etoposide clearance accounts for 30%-40% of total

plasma clearance (1.44 L/h/m^2) however, biliary excretion is very small (2%) (Yarbro (ed.), 1992).

Several etoposide metabolites have been confirmed or postulated, such as hydroxy acid metabolite identified in urine samples (accounts for <2% of an administered dose), picro lactone derivatives and aglycone which has not been identified in clinical samples (Yarbro (ed.), 1992). Formation of these metabolites could have potential importance since the hydroxy acid and picro lactone derivatives are considerably less cytotoxic than the parent drug, while aglycone retains cytotoxicity. The aglycone was reported to arrest cells in mitosis but it did not produce any significant effect on DNA, RNA or protein synthesis nor on DNA breakage (Anderson *et al.*, 1994). Another etoposide metabolite, the glucoronide conjugate, has been identified and a mean of 27% of administered etoposide drug was found excreted in the urine as etoposide glucoronide (Yarbro (ed.), 1992).

2.11 Mutagenicity.

2.11.1 Mutagenicity in non-mammalian cells.

Mutagenicity of etoposide in prokaryote systems is weak or negative. The *Salmonella* mutagenicity assay, T4 phage rFCII gene reversion assay, and prophage induction assays have been used most extensively for the evaluation of etoposide and other topoisomerase II inhibitors (Anderson *et al.*, 1994). Etoposide showed no increase in revertant frequencies in the *S. typhimurium* TA98 strain and in the base-substitution tester strain TA100 in either the presence or absence of an exogenous rat liver activation system. A very weak mutagenic response (<2-fold increase in revertant frequency) was seen at very

high drug doses in the TA102 strain that is sensitive to agents that cause single-strand breaks in DNA. Etoposide has been reported to be more mutagenic in the *S. typhimurium* uvrB$^+$ frameshift strains UTH8413 and TA 1978 than in their uvrB$^-$ (nucleotide excision repair-deficient) counterparts TA98 and TA1538, respectively, though at very high doses (2000 μg/ml) (Matney *et al.*, 1985). Etoposide also failed to show any mutagenic response in an excision-repair proficient *E. coli* strain 113/143 employing two different forward mutation detection systems that are capable of detecting various types of genetic lesions (Gupta *et al.*, 1987). No mutagenic response to etoposide was seen in *E. coli* strain WP44SNF under conditions where error-prone repair was either induced or not induced (Gupta *et al.*, 1987). The lack of mutagenic response by etoposide in the reversion assays may be due to the fact that these targets are unable to detect the specific genetic damage that etoposide induce *i.e.*, DNA-strand breaks. However, systems in *E.coli* should detect all types of genetic lesions. The lack of mutagenicity correlated with no significant cellular toxicity in the prokaryotic systems indicating that etoposide does not produce significant genotoxic damage in the bacterial cells.

Contrary to etoposide, other topoisomerase II inhibitors such as acridine and ellipticine have demonstrated mutagenicity in Ames' *Salmonella typhimurium* frameshift tester strains, and their mutagenicity has been explained by intercalation into, and/or covalent adduct formation with DNA (DeMarini *et al.*, 1983, Ferguson *et al.*, 1985). It was postulated that bacterial systems have the capacity to reflect genotoxicity mediated by mechanisms other than target-drug interaction in mammalian cells, such as DNA intercalation, DNA-adduct formation, oxygen-radical generation and interaction with

endogenous bacterial topoisomerase II enzymes (Anderson *et al.*, 1994). Etoposide does not intercalate DNA and, although it was shown to produce hydroxyl free radicals, these radicals were shown not to be able to react with molecular oxygen or DNA (Kalyanaraman *et al.*, 1989). Consequently, these radicals are not mutagenic contrary to oxygen-radical-mediated DNA damage that accounts for frameshift mutagenesis by m-AMSA, the anthracyclines and ellipticine derivatives.

The lack of or weak mutagenicity of etoposide in bacterial systems suggests that the topoisomerase II enzymes in prokaryotic and mammalian cells could differ in their interaction with the drug. Despite the dissimilarities of the two enzymes (Gupta *et al.*, 1987), etoposide has been reported to poison the bacterial DNA gyrase of *E.coli* (Liu 1989). However, the *Salmonella* assay seems to be a poor indicator of DNA gyrase mediated genotoxicity since the quinolone class of agents, which also stabilize cleavable complexes of DNA gyrase, are only weakly mutagenic in the *Salmonella* assay (Anderson *et al.*, 1994). They are, however, more potent as prophage inducers (Anderson *et al.*, 1994), thus confirming their capacity to mediate DNA strand break induction *in vivo*. This suggests that frameshift and base-substitution reversion in the *Salmonella* assay may be inappropriate endpoints for gyrase-mediated genotoxicity. However, the lack of mutation induction by etoposide in *E.coli* 343/113 system which is capable of detecting all types of genetic lesions is unresolved.

The phage T4 topoisomerase II is unique because both DNA gyrase and eukaryotic topoisomerase II-specific agents stabilize its cleavable complexes (Huff *et al.*, 1990). However, while *m*-AMSA is a potent frameshift inducer in the bacteriophage T4, etoposide failed to significantly revert T4 rIIB gene frameshifts (Huff *et al.*, 1990). The

mutagenicity of *m*-AMSA in this system likely involves T4 topoisomerase-II-*m*-AMSA interaction, because the majority of *m*-AMSA induced frameshifts, which revert the T4 rIIB gene, occur at the same sites where *m*-AMSA potentiates T4-topoisomerase-II cleavable complex formation in the rIIB gene *in vitro* (Ripley *et al.*, 1988). Several reasons for the lack of mutagenicity by etoposide in this system were proposed. It is possible that the sites of etoposide and teniposide stabilized cleavable complexes lie outside the target region for rIIB frameshift reversion suppression. Alternatively, etoposide and teniposide may potentiate a different form of topoisomerase II genotoxicity such as large DNA deletions, which would not score in the T4 reversion assay. Epipodophyllotoxins might also stabilize cleavable complexes in a manner that prevents polymerase or exonuclease from contributing to frameshift induction (Anderson *et al.*, 1994). Alternatively, frameshift mutagenesis in T4 may not be a characteristic property of topoisomerase II poisons and m-AMSA may be somewhat different from epipodophyllotoxins in this respect.

Etoposide was also non-mutagenic in forward- and reverse-mutation tests in the lower eukaryote *Neurospora crassa* and a similarity between the topoisomerase II of *N.crasa* and DNA gyrase of bacteria was suggested (Gupta et al., 1990).

2.11.2 Mutagenicity in mammalian cells.

Contrary to non-mammalian systems etoposide and teniposide were found to be highly mutagenic in some mammalian cells. Both drugs were strongly mutagenic in CHO cells and in mouse L cells as indicated by the induction of mutation in the *hprt* and adenosine kinase (*ak*) loci (Gupta *et al.*, 1987, Singh and Gupta, 1983). Teniposide was 10-fold

more potent in cytotoxicity and mutagenicity. In mouse L cells 16h exposure to 1.5 μg/ml of etoposide reduced cell survival to less than 5% of the control and 0.5-1μg/ml of etoposide increased *hprt* MF by more than 15-fold over the background MF. Concentration-dependent DNA fragmentation was also detected by sedimentation on alkaline sucrose gradient. Similar responses were seen in CHO cells (Singh *et al.*, 1983). AK like HPRT is a nonessential purine salvage pathway enzyme, and should detect the types of genetic lesions similar to those detected by the *hprt* genetic target. While the same for etoposide, tenoposide showed a more pronounced effect at the *hprt* than at the *ak* locus suggesting the differential accessibility or affinity of specific chromosomal regions corresponding to these loci by teniposide. Singh and Gupta (1983) also studied mutagenic responses of 13 anticancer drugs including etoposide on mutation induction at five genetic loci in CHO cells. Only one locus, *hprt*, was not essential for cell survival and was expected to select mutants with all types of genetic damage, while others were specific for only certain types of DNA changes (base substitutions) which would lead to resistance without destroying the protein function. Again, both etoposide and teniposide were strongly mutagenic at the *hprt* locus, weakly mutagenic at the emetine and DRB, and non-mutagenic at the *oau* and *mbg* loci. After 16 h etoposide exposure at a concentration of 1 ug/ml the cell survival was 40% and the *hprt* MF/10^6 cells increased from 1.28 to 28 and reached 46 at 1.25 μg/ml of etoposide with 27.5% survival. Also, all drugs significantly increased the frequency of SCE, and a very good correlation was observed between the SCE frequency and the mutagenic responses at the *hprt* locus. Thus, etoposide and teniposide were particularly potent in both of these assays. It was

proposed that etoposide and teniposide produce predominantly those types of genetic alterations which do not result in specific single base substitutions (frameshifts, deletions, strand breaks, chromosomal rearrangements).

Consistent with the previous results, Berger *et al.* (1991) found that etoposide induced partial deletions and/or rearrangements of the *hprt* gene in Chinese hamster V79 cells. They exposed the cells to 5 and 10 μM etoposide and compared the spectrum of mutations found in 3 spontaneous and 10 etoposide-induced mutants by Southern blot hybridization analysis. Genomic DNA digested with three different restriction enzymes was hybridized to hamster *hprt* cDNA. The results showed that while spontaneous mutants had "normal" pattern of hybridization or negligible deletions or rearrangements, six etoposide-induced mutants had completely lost the *hprt* gene and the rest four mutants had gross deletions and/or rearrangements.

Han *et al.* studied teniposide-induced mutations at the hemizygous adenosine phosphorybosil transferase (*aprt*) locus of CHO D422 cells which were analyzed by PCR and sequencing (Han *et al.*, 1993). They found that teniposide increased the frequency of the *aprt* and *hprt* mutations in a dose-dependent manner. At low concentrations (0.02-0.16 μg/ml), mutagenesis at the *aprt* gene increased but rapidly reached plateau and the induced MF was 7-fold greater than the spontaneous frequency. The *hprt* MF increases (15-fold) were consistent with similar studies by Singh and Gupta (1983). The induced spectra of 65 mutations differed from the spontaneous spectra of 42 mutations. However, contrary to Berger *et al.* (1991), the majority of induced mutations (63%) were deletions, duplications and insertions of various sizes, with the majority being less than 20 bp.

While the number of small deletions did not change, the number of insertions, mostly duplications increased in the induced spectra from 7% to 23%. The number of large deletions or rearrangements increased from 0 to 11%, and 7 mutants in the induced spectra exhibited the loss of PCR products indicative of large-scale deletions, disruptions or rearrangements confirmed by Southern blotting hybridization analysis. The remaining mutations were base substitutions, the majority of which were transversions, whereas equal numbers of transitions and transversions were recovered in the spontaneous spectra. Two induced mutants, but none of the spontaneous mutants had complex mutations consisting of two deletions and an insertion in one case, and complex duplications/rearrangements in the other. In this study, Han *et al.* (1993) also examined the sites of drug-simulated cleavage mediated by topoisomerase II *in vitro*. They found that about 70% of the sites were apparent double-strand cleavage sites, as indicated by the 4-base stagger between breaks in complementary strands. There was also a highly significant tendency for the strong cleavage sites to have a C at the -1 position, consistent with *in vitro* teniposide-stimulated cleavage of SV40 DNA and DNA of the *c-myc* proto-oncogene. Significant correspondences were found between the sites of teniposide-induced small deletions and, to a lesser extent, duplications and strong cleavage sites mediated by topoisomerase II. Sequences which were deleted showed a much higher incidence of strong cleavage sites within them than did sequences not involved in the deletions, however, the exact positioning of the cleavage sites with respect to the deletion termini was variable.

Three models were proposed which could explain the formation of most of the mutations induced by teniposide (Han *et al.*, 1993): a) double-strand break repair by non-

homologous end-joining; b) replication slippage misalignment; and c) addition or deletion of a few nucleotides at the free 3' ends left by the topoisomerase II. Later, Ripley (1994) proposed a unifying modified nick-processing model to explain the mechanism of deletions and duplications formation induced in CHO cells by teniposide. Ripley applied the nick-processing model that explained the formation of mutations by acridines (*m*-AMSA) in bacteriophage T4. According to this model, frameshift mutations originate due to the ability of these mutagens to cause DNA cleavage by the topoisomerase II of T4 and the subsequent processing of the 3' ends at DNA nicks by DNA polymerase or its associate 3' exonuclease followed by the ligation of the processed end to the original 5' end. The difference between the T4 and CHO cells is that there was an absolute maintenance of the 5'-PO_4 end of a nick in T4 while such integrity was lost in 80% of CHO teniposide-induced mutants. To explain mutations in CHO cells, Ripley (1994) proposed a modified nick-processing model which postulates that either 3' ends at nicks are elongated by DNA polymerase and/or that 5' ends of nicks are subject to nuclease activity (3'-nuclease activity is not implicated). Interestingly, teniposide treatment of T4 revealed no induction of frameshifts (DeMarini *et al.*, 1988) and the specificity of processing of teniposide-induced breaks seen in CHO cells was proposed to explain the absence of teniposide-induced mutagenesis in T4.

Etoposide was found to induce dose-dependent increase in the *hprt* mutations in Chinese hamster V79 cells at concentrations ranging from 0.3 to 0.8 μM (Hashimoto *et al.*, 1995). Interestingly, the suppression of apoptosis in these cells by bcl-2 overexpression led not to the increase but rather to the decrease in MF induction by the

drug. This was consistent with the model of etoposide cell killing according to which etoposide-induced genetic recombination processes are responsible for the induction of mostly large deletions and rearrangements in induced mutational spectra and for the etoposide-induced cytotoxicity. The reduction in cytotoxicity then would parallel the reduction in the genetic recombination and in the MF.

The ability of etoposide and teniposide to induce primarily large deletions and rearrangements is further supported by the fact that the frequency of drug-induced mutations at a heterozygous thymidine kinase (*tk*)+/- locus in L5178Y/TK+/-3.7.2C mouse lymphoma cells is about 30-fold higher than that at a functionally hemizygous *hprt* locus (DeMarini *et al.*, 1987, Ashby *et al.*, 1994). The induction of small-colony growth mutants in L5178/TK+/- indicates that teniposide induced large deletions and rearrangements. Such mutations will not affect the survival of L5178/TK+/- cells as the essential genes flanking the heterozygous *tk*+/- locus in mouse lymphoma cells are expressed by alleles present on both copies of chromosome 11 where *tk* resides. Large deletions and rearrangements of the *hprt* gene on the other hand will lead to the inactivation of essential genes which may be adjacent to the *hprt* gene located on the only expressed allele on the X chromosome and the mutant cells will not survive. Interestingly, 83% and 92% of large and small colony *tk* mutants, respectively, had lost the entire target gene sequence (Ashby *et al.*, 1994).

The induction of small colony *tk* mutants by teniposide may occur by the same mechanism that mediates chromosomal aberrations or SCE. Each of these processes involves DNA exchanges or deletions comprising multiple genes. Support for this hypothesis is provided by the observation of teniposide-induced DNA fragments isolated

under protein-denaturing conditions from Chinese hamster fibroblasts analyzed by field inversion gel electrophoresis (Razin *et al.*, 1991). These fragments were up to several hundred kilobases in length. One particular fragment was found to contain the α-globin gene cluster and the DNA sequences at the ends were characteristic of nuclear matrix attachment regions (MARs). Genomic DNA organized as multiple gene containing loops is thought to be affixed to the nuclear matrix at MARs and associated there with topoisomerase II (see above). Large DNA deletions, therefore, may result from processing of drug stabilized cleavable complexes occurring at MARs.

Further support for the MARs hypothesis is provided by the genetic marker studies in the AL system (CHO cells containing single human chromosome 11, which expresses a selectable cell surface antigen). In this system and intercalating topoisomerase II-inhibitor *m*-AMSA was found to induce deletions of at least 1.5-2 megabase pairs in length (Shibuya *et al.*, 1994). To explain the observed large deletions the authors proposed the topoisomerase II-subunit exchange mechanism which involves the exchange between topoisomerase II molecules in separate chromosomal minibands or may involve a series of exchanges between many topoisomerase II-DNA loop complexes or replicon loops (Figure 1.6). Each replicon loop is 0.05-0.1mbp in length and is fixed to the nuclear matrix at MARs where topoisomerase II is thought to interact with DNA while each miniband is comprised of multiple loops (17 loops or 1mbp in length). The multiple subunit exchanges between a series of loops or fewer subunit exchanges between minibands will result in the observed 1.5-2 mbp deletions.

2.12 Apoptosis and mutagenicity.

While it is assumed that apoptosis eliminates damaged cells from population, several reports indicate that treatment of cells with topoisomerase II inhibitors at concentrations that induce apoptosis also results in mutation induction. AHH-1 human lymphoblastoid cells exposed to m-AMSA underwent delayed apoptosis and at the same time a significant dose-dependent increase in the mutation induction at the *tk* gene was observed (Morris *et al.*, 1995). Consistent with other reports, the mutation induction at the *tk* locus was primarily due to the recovery of mutants with the small colony or slow growth phenotype indicative of large, intergenic multi-locus deletions and rearrangements. Induction of the *hprt* mutants was much less and was insignificant or reached significance depending on the statistical test (Morris *et al.*, 1995). Thus, cells with DNA damage can either escape or suppress the signals for apoptosis.

Apoptosis is thought to play a crucial role in maintaining genomic integrity by selectively eliminating highly mutated cells from the population. If this were true then the suppression of apoptosis would result in the enhanced mutagenesis or mutation frequency due to an increase in clonogenic survival of the mutant cells. However, contrary to this hypothesis, suppression of etoposide-induced apoptosis in V79 cells produced by the overexpression of human *bcl-2* gene led not to the increase but to the reduction of the *hprt* MF (Hashimoto *et al.*, 1995). This result was consistent with a proposed mechanism of etoposide cytotoxicity where both the cell death and mutagenicity result from the etoposide-induced illegitimate recombination (Figure 1.5). According to this scheme, suppression of cell death would parallel the suppression of mutagenesis since the source of these two events, genetic recombination measured by the SCE frequency, was substantially diminished in the *bcl-2* overexpressing cells. Therefore, according to these

experiments, *bcl-2* overexpression suppressed etoposide-induced apoptosis not by permitting highly mutated cells to survive but rather by preventing mutagenesis and consequently apoptosis itself through the inhibition of etoposide-induced recombination. Contrary to the effect of etoposide, the *bcl-2* overexpression in TK6 cells resulted in the suppression of radiation-induced apoptosis and in the enhanced radiation-induced mutagenesis (Cherbonnel-Lasserre *et al.*, 1996). Therefore, the nature of the cell line and of the genotoxic agent seems to affect the mechanism of how *bcl-2* overexpression prevents apoptosis.

Factors that contribute to the apoptosis resistance may include mutations in apoptosis genes such as *p53*, *bcl-2* and others. *p53* status alone does not predict apoptosis by topoisomerase II inhibitors since Solary *et al.* (1994) demonstrated apoptosis induction in HL-60 cells, despite them not having a functional *p53* gene (see above). Apoptosis induction also seems to be agent and cell type specific. There is evidence that CHO cells, although susceptible to apoptosis by cisplatin, are not prone to apoptosis following treatment with topoisomerase II poisons (Cortes *et al.*, 1994, Demarcq *et al.*, 1994). In contrast, human peripheral blood lymphocytes and various human leukemia and lymphoblastoid lines appear especially prone to apoptosis by topoisomerase II poisons (Morris *et al.*, 1995, Fernerdes *et al.*, 1994).

2.13 Chromosomal effects.

2.13.1 Aberrations

There may be many pathways by which drug-stabilized complexes can lead to chromosomal aberrations, however, it appears that additional disruptive processes such as replication, transcription, repair or cell division are necessary to convert the drug-

stabilized complexes with maintained strand continuity into chromosomal breaks and rearrangements.

Huang *et al.* (1973) found that continuous etoposide exposure caused a high incidence of cells with chromosomal aberrations which were of chromatid type and were induced in the G2 and S phase of the cell cycle in four human hematopoietic cell lines. The induction of chromosome type aberrations (mostly breaks and exchanges) by etoposide was found in Chinese hamster lung fibroblasts and the induction of aberrations was associated with the stabilization of the cleavable complexes by Topo II (Suzuki *et al.*, 1995). Chromosomal aberrations were also observed after etoposide treatment in Chinese hamster DC3F cells (Pommier *et al.*, 1988) and in human ovarian cancer cells (Noviello *et al.*, 1994) where correlation between the drug-induced aberrations and double-strand breaks was observed. Lymphoblastoid lines derived from AT patients are hypersensitive to agents that produce double-strand breaks. These lines were hypersensitive to the chromosomal aberrations by etoposide indicating that double-strand breaks have an important role in etoposide clastogenic and cytotoxic activity (Caporossi *et al.*, 1993).

Chromosomal aberrations were also observed in human lymphocytes at concentrations as low as 0.025 μg/ml (Tominaga *et al.*, 1986). The aberrations were generally of the chromatid type such as breaks, exchanges, deletions and gaps, but were sometimes of the chromosome type, such as dicentrics and rarely tricentrics, and ring chromosomes. There was no correlation between the sites of chromatid deletions and the sites of SCE, suggesting that chromatid deletions induced by etoposide derived from unrepaired chromatid breaks rather than incomplete exchanges. Predominantly chromatid type

aberrations specific for S-phase cells were detected in Swiss albino mouse bone marrow after *in vivo* etoposide exposure of the mice (Agarwal *et al.*, 1994).

It was proposed that the mechanism of chromosomal aberrations might involve limited endonuclease activation such as that seen in apoptosis (Anderson *et al.*, 1994). Endonucleases can cause double-strand breaks which would, if generated prior to replication, lead to chromosome-type aberrations and, if generated after the round of DNA replication, would generate chromatid-type lesions.

The clastogenic effect of etoposide has been investigated *in vitro* in lymphocytes of 5 healthy donors and non-random involvement of chromosomes 1, 11, and 17 in aberrations was detected (Maraschin 1990). These chromosomes are R-band-rich with actively transcribed genes, and the authors suggested that etoposide clastogenicity might be more pronounced at these chromosomal regions.

2.13.2 SCE.

Etoposide was shown to induce concentration-dependent SCE both *in vitro* and *in vivo* (Pommier *et al.*, 1988, Noviello *et al.*, 1994, Tominaga *et al.*, 1986, Agarwal *et al.*, 1994). Cleavable-complex stabilization is also the main mechanism by which etoposide lead to induction of SCE. As with other types of DNA damage SCE induction by etoposide requires processes active during the S phase of the cell cycle (Dillehay *et al.*, 1987). However, contrary to chromosomal aberrations, a strong correlation has been observed for the induction of SCE and cytotoxicity (Anderson *et al.*, 1994, Noviello *et al.*, 1994). It was proposed that topoisomerase interactive agents promote non-reciprocal exchange of DNA between sister chromatids which causes cells to die by disrupting or deleting essential genes (see above, Figure 1.5). In support for the ability of

topoisomerase II to mediate strand exchange, a number of studies have demonstrated that end-points of drug-induced DNA recombinational events coincide with sites of drug-induced topoisomerase II cleavable complex stabilization (Bae *et al.*, 1988).

2.13.3 Recombination and effects on segregation at mitosis.

Charron and Hancock (1991) found that segregation of the daughter genomes during mitosis was frequently aborted in CHO cells grown with teniposide. This phenomenon was explained by the frequent formation of quadriradial chromosomes, which result from reciprocal exchange of double stranded DNA between single chromatids of two different chromosomes. These chromosomes recombined between their centromeres give rise to chromatids bearing translocations and containing either two or no centromeres and kinetochores and these would fail to segregate properly on the mitotic spindle, giving rise to the formation of micronuclei. The authors interpreted their observations as evidence that topoisomerase II can mediate chromosome recombination *in vivo*. This process occurred after and independent of DNA replication by a proposed mechanism of topoisomerase II homodimer subunit exchange.

The same mechanism is proposed to explain the topoisomerase II involvement in illegitimate recombination linked to dysfunction of chromosomal loop attachment sites at MARs where topoisomerase II operates (Ikeda et al., 1994). Bodley *et al.* (1993) found that integration of simian virus 40 into cellular DNA occurs at or near topoisomerase II cleavage hot spots induced by teniposide.

Tominaga *et al.* (1986) observed tetraploid cells with or without endoreduplication (an internal doubling of chromosomes resulting from two successive DNA synthesis period without intervening cytokinesis which is considered to be associated with the etiology of

polyploidy) in human lymphocytes treated with etoposide suggesting that etoposide has an effect on division process or chromosome segregation.

Etoposide treatment of neonatal lymphocytes induced only a very small increase in the number of micronuclei containing whole chromosomes even at high drug concentrations (Slavotinek *et al.*, 1993). Such micronuclei are thought to arise as a result of non-disjunction leading to aneuploidy. This observation suggests that segregation abnormalities are less important than other mechanisms in etoposide cytotoxicity in these cells.

Mailhes *et al.* (1994) found that etoposide induced aneuploidy in mouse oocytes. Zucker *et al.* (1991) reported that murine erythroleukaemic cells did not form mitotic figures after 4h exposure to clinically achievable levels of teniposide and instead, the authors showed a time-dependent increase in the proportion of cells with 4N and up to 8N DNA. They suggested that interference with chromosome condensation during G2-phase led to the induction of polyploidy in these cells.

2.14 Differentiation, gene amplification and other genotoxic effects.

Etoposide has been shown to induce differentiation of U937 promonocytic cells (Perez *et al.*, 1994 (1)). At drug concentration leading to a little cell damage the treatment induced a transient increase in *c-fos*, *c-jun*, and *jun B* mRNA levels, allagenase mRNA level, and a progressive increase in vimentin and lamin A and C mRNAs. Etoposide also caused effects on protein kinase C and AP-1-dependent gene expression.

Schonfield *et al.* (1992) found that teniposide induced hypomethylation of DNA and differentiation in a number of cell lines. They suggested that altered topoisomerase II activity occurring during the process of tumor progression may provide a link between

induction of polyploidy, DNA hypomethylation and aberrant gene expression frequently observed in tumor cells.

The inhibition of topoisomerase II by etoposide caused gene amplification in Chinese hamster V79/B7 cells which was correlated with the frequency of chromosomal aberrations (Leonardo *et al.*, 1993) consistent with the idea that chromosomal breakage and recombination events are a prerequisite for generating amplified DNA sequences in mammalian cells. This inhibition could represent the basis of the gene amplification-mediated cross-resistance to unrelated drugs seen in tumor cells treated with antitumor drugs that specifically target topoisomerase II (Leonardo *et al.*, 1993).

The involvement of etoposide in DNA repair has been demonstrated by Thielmann *et al.* (1993) who showed that etoposide strongly suppressed repair-specific DNA excision following UV-irradiation of human fibroblasts.

Induction of micronuclei was demonstrated in male rat spermatogenic cells and in cultured rat seminiferous tubules during meiosis (Lahdetie *et al.*, 1994), in the mouse bone marrow micronucleus assay (Ashby *et al.*, 1994) and in neonatal lymphocytes (Slavotinek *et al.*, 1993).

2.15 Reproductive toxicology.

Etoposide and teniposide were implicated in germ-cell genotoxicity. Hakovirta *et al.* (1993) studied the effects of etoposide on stage-specific DNA synthesis during rat spermatogenesis. They found that etoposide inhibited premitotic DNA synthesis at specific stages, but that the pre-meiotic DNA synthesis was less effectively inhibited. Wright and Schatten (1990) studied the effects of teniposide on nuclear events during oocyte maturation, fertilization, and early embryonic development of fertilized *Spisula*

solidissima oocytes, using DNA fluorescence. They found a stage-specific inhibition of chromosome separation and block of chromosome condensation.

The reproductive toxicology of etoposide has been demonstrated in a variety of assays in rodents (reviewed in Anderson *et al.*, 1994). Etoposide was shown to produce measurable genotoxicity in spermatogonia and in embryo, to cause abnormal fetus and offspring development and to decrease survival rate of progeny. Experience in humans demonstrates that etoposide can impair female fertility. Etoposide has been associated with spontaneous abortions, female infertility and congenital abnormality in offspring (Anderson *et al.*, 1994).

2.16 Secondary leukemias after epipodophyllotoxin chemotherapy and translocations involving MLL gene on 11q23.

Epipodophyllotoxins are used in chemotherapy, for the most part, in combination with DNA-alkylating agents such as cis-platin and cyclophosphamide, therefore, the incidence of malignancy induced by etoposide or teniposide given as single agents is unknown. A variety of malignancies, however, including AML, non-small cell lung cancer (NSCLC), breast and prostate cancer, squamous cell cancer of the head and neck and basal cell cancer of the skin, have been reported after the use of etoposide combined with cis-platin to treat SCLC (Anderson *et al.*, 1994).

AML has been reported at high frequency in cohorts of patients receiving epipodophyllotoxins in combinations with agents of low leukemogenic potential (reviewed in Anderson *et al.*, 1994). The occurrence of sAML is especially striking after use of etoposide combined with cis-platin for NCSLC (cumulative risk 44%±24% at 2.5

years, mean etoposide dose 6480 mg/m^2), in children treated with etoposide among other drugs for NHL (cumulative risk 18.4%±3.2% at 4 years, mean etoposide dose 5160 mg/m^2) and after chemotherapy including epipodophyllotoxins for childhood ALL (cumulative risk 12.4% at 6 years, mean etoposide dose 7371 mg/m^2 plus mean teniposide dose of 4636 mg/m^2) (Anderson *et al.*, 1994).

The risk of sAML is dependent not only on the total dose of etoposide and/or teniposide received but also on the administration schedule. Thus, most cases of AML occurred in patients who received at least 2000 mg/m^2 of etoposide and/or teniposide. The germ cell tumor patients who received a mean of 2000 mg/m^2 or less of etoposide had the cumulative sAML risk of 0 or 0.37% at 2.3 years, while a cohort of germ cell tumor patients treated with a mean of 3086 mg/m^2 of etoposide had 4.7%±2.3% cumulative risk of sAML at 5.7 years. At the same time, the cumulative sAML risk in children with ALL treated with similar doses of etoposide and/or teniposide was much lower in those patients who received the drugs every other week compared to the weekly schedule (1.6% and 1.1 % *vs.* 12.4% and 12.3% respectively). Epipodophyllotoxins are strongly implicated in the etiology of sAML in these studies because no cases of sAML were reported in groups of germ cell patients in which vinblastine was used instead of etoposide with bleomycin and cis-platin (Anderson *et al.*, 1994).

The chromosomal abnormalities in leukemias arising after combination chemotherapy with epipodophyllotoxins (balanced translocations involving bands 11q23 and 21q22) differ from non-balanced abnormalities of chromosomes 5 and 7 most frequently seen in

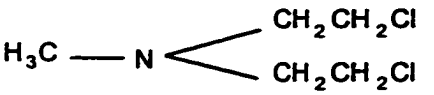
leukemias arising after alkylating agent therapy and are consistent with the mechanism involving topoisomerase II.

Chromosomal rearrangements involving 11q23 are observed in more than 50% of all epipodophyllotoxin-related leukemias and are also seen in spontaneously occurring leukemias, especially in childhood ALL and AML (Felix *et al.*, 1995 (1)). Involvement of 11q23 in leukemias is an indicator of poor prognosis (Braekeller *et al.*, 1986). Most of the 11q23 breakpoints in spontaneously arising leukemia and in the cases of epipodophyllotoxin-induced sAML cluster in the *MLL* gene (Bernard *et al.*, 1995). Other cytogenetic abnormalities are observed less consistently. The *MLL* (*ALL-1*, *HRX-Drosophila Trithorax* homolog) gene and its 8.3 kbp breakpoint cluster region (bcr) have been characterized (Chapter V).

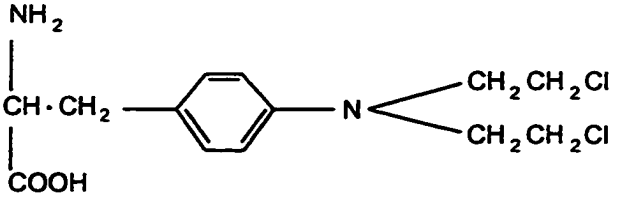
3. Genetic profile of alkylating agent melphalan.

3.1 Chemical structure, history and current use.

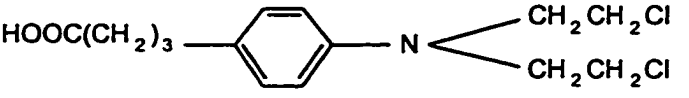
Melphalan (L-phenyl-alanin mustard, L-PAM/PAM, Alkeran) is a bifunctional alkylating agent (Figure 1.7). Alkylating agents have been used therapeutically for decades. Sulphur mustard was first synthesized in 1854 and its hazardous biological effects have been described by 1919. Krumbhaar *et al.* (1919) noticed that mustard "gas" (di(2-chloro-ethyl)sulphide (in fact a poison oil) used in the 1914-1918 War, caused death through massive damage to haematopoietic system. The American military had made other "war gases" analogous to mustard gas by substituting nitrogen for sulfur, or "nitrogen mustard gases", typified by HN_2, di(2-chloroethyl)methylamine (mechlorethamine) (Figure 1.7). Soluble in saline, nitrogen mustards were introduced into therapeutic use at the end of the Second World War (Gilman *et al.*, 1946). Specific action



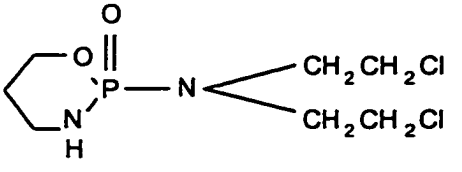
Mechlorethamine (NH_2)



Melphalan



Chlorambucil



Cyclophosphamide

Figure 1.7 Chemical structures of clinically useful nitrogen mustards.

on the bone marrow suggested a possible use of nitrogen mustards in cancer chemotherapy against lymphomas and leukemias (Goodman *et al.*, 1946). Nitrogen mustard gas was shown "capable of breaking chromosomes and thus interfere with mitosis" (Auerbach *et al.*, 1946). Biesele *et al.* (1950) have shown that chromosome breakage in cells treated with nitrogen mustards was due to crosslinking alkylation and recognized the difference between the mono- and polyfunctional alkylating agents. The observation that monofunctional alkylating analogues of mustards were much less effective cytotoxic agents prompted Goldacre *et al.* (1949) to propose that "cross-linking of macromolecules involved in cell division was essential for cytotoxicity of alkylating agents".

Interest in chemical carcinogens as potential anticancer agents prompted the development of nitrogen mustards derived from aromatic amines including melphalan at the Chester Beatty Research Institute (Bergel *et al.*, 1954). Melphalan is currently primarily used against multiple myeloma, breast and ovarian cancer.

3.2 Intracellular targets and mechanism of action.

The cytotoxic action of melphalan is related to its ability to transfer alkyl groups to cellular constituents, which may interfere with mitosis and cell division. Melphalan reacts spontaneously to produce a positively charged carbonium ion or a transition complex capable of forming a covalent bond with a nucleophilic centre (Povirk *et al.*, 1994) (Figure 1.8). Nucleophilic sites are commonly found in macromolecules and include phosphate, amino, sulfhydryl, hydroxyl, carboxyl and imidazole groups found in DNA, RNA and proteins. The nucleophilic centers on DNA include guanine-N7, adenine-N1 and N3, cytosine-N3, guanine-O6 and the phosphates between the bases (Ludlum, 1975).

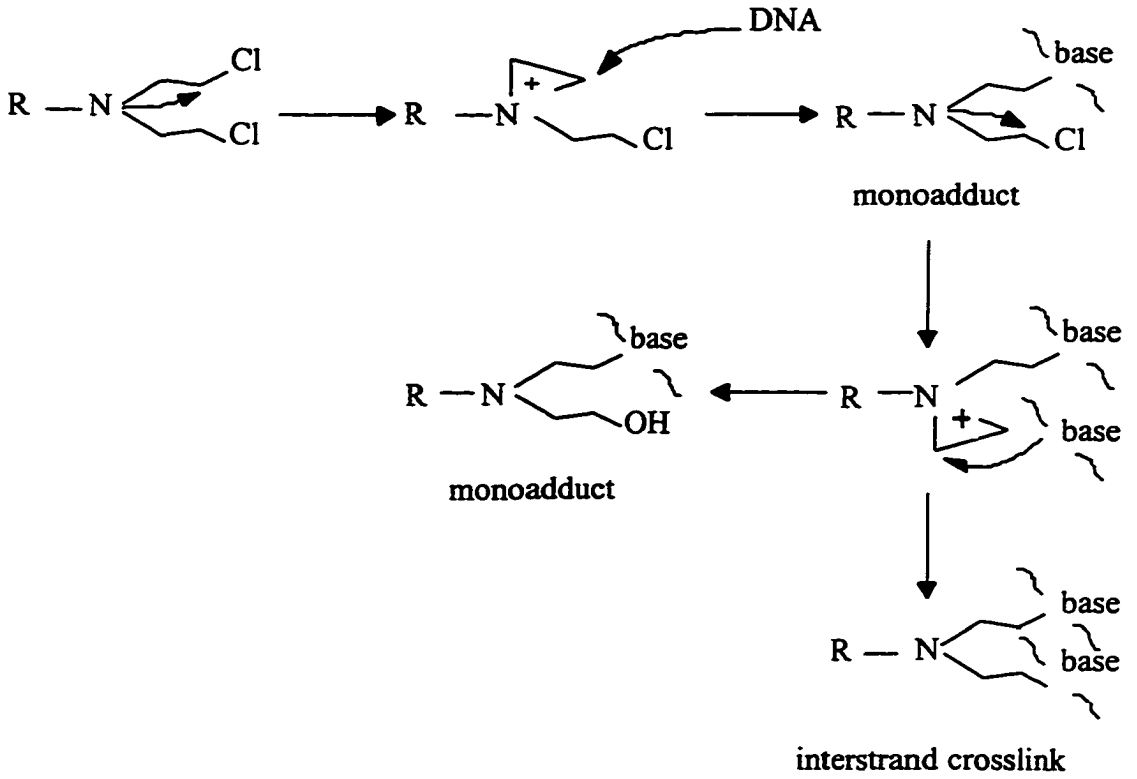


Figure 1.8 Generalized scheme for the reactions of nitrogen mustards with DNA. (Adapted from Povirk *et al.*, 1994.)

The N7 position of guanine in DNA is particularly susceptible to alkylation because it has one of the lowest molecular electrostatic potentials coupled with low steric restriction of the site. The major site of crosslinking is assumed to be between two guanine N7 positions, and the reactivity of different guanine N7 sites has been shown to be influenced by the molecular electrostatic potential of neighboring base pairs (Kohn *et al.*, 1987). Melphalan is a bifunctional alkylating agent because it has two reactive groups and it can form monoadducts as well as inter- and intra-molecular cross linkages between strands of DNA or between DNA and proteins. Reaction of melphalan with DNA proceeds via initial formation of a non-covalently bound monoadduct (Figure 1.8). The monoadduct can then undergo one of three further reactions: reaction with an external nucleophile such as solvent or protein, reaction with a base on the same DNA strand to give an intrastrand crosslink, or reaction with a base in the opposite strand to give an interstrand crosslink (Figure 1.8) (Povirk *et al.*, 1994).

Melphalan readily forms N7 adducts with guanine nucleosides and nucleotides (38% of total DNA alkylation) and crosslink adducts between two guanines (20% of total DNA alkylation) (reviewed in Povirk *et al.*, 1994). Melphalan also forms a relatively high proportion of adenine adducts, whose properties are consistent with alkylation at the N3 position (20% of total DNA alkylation) as well as guanine N7- adenine-N3 crosslinked adducts (13% of total DNA alkylation) (Povirk *et al.*, 1994). Thus, about 90% of products from alkylation of DNA by melphalan are accounted for by four principal alkylpurines: A-M-OH, A-M-G, G-M-OH or G-M-G. The A-M-G adduct makes much greater demands on DNA conformation than the G-M-G adduct since guanine-N7 faces the major groove and adenine-N3 faces the minor groove. The considerable distortion of

the double helical structure of DNA and the finding that adenine adducts are formed preferentially at A/T-rich tracts of DNA, make it difficult to explain the relative abundance of A-M-G adducts. It was proposed that the formation of the A-M-G adducts is related to the initial preferential alkylation at N3 of adenine following relatively selective binding of melphalan to the minor groove or by a reaction of a depurinated monofunctional adduct with a second DNA molecule (Povirk *et al.*, 1994). Melphalan-induced interstrand crosslinks between two guanines occur at GNC sequences, which would also require a significant deformation of the DNA conformation and the mechanism of this is yet unclear (Bauer *et al.*, 1994).

3.3 Mechanism of cytotoxicity and repair of melphalan-induced DNA damage.

The relatively weak cytotoxicity of monofunctional mustards strongly suggests that bifunctional lesions are responsible for cell death (Povirk *et al.*, 1994). The primary cause of cell death by melphalan is believed to be due to the interstrand crosslinks, however, the possibility that other bifunctional lesions (intrastrand or DNA-protein crosslinks) could also play a role in cytotoxicity is not excluded.

The cytotoxicity of melphalan is associated with DNA interstrand crosslinking ability and guanine-N7 alkylation (Sunters *et al.*, 1992). With regard to the possible cytotoxic effect of DNA adenine alkylation by melphalan, it should be noted that the resistance to melphalan in chronic lymphocytic leukemia patients was associated with increased activity of 3-methyladenine-DNA glycosylase, a repair enzyme that removes 3-alkyladenines from DNA (Geleziunas *et al.*, 1991).

Early studies with bacteriophage T7 demonstrated that a single nitrogen mustard-induced interstrand crosslink was lethal to the bacteriophage (Verly *et al.*, 1969),

however, both intra- and interstrand crosslinks were implicated as being lethal lesions in T7 induced by sulphur mustard gas (Lawley *et al.*, 1969). Studies with *E. coli* and mammalian cells revealed that both nitrogen mustard monofunctional and bifunctional adducts, as well as DNA-protein crosslinks are repaired in these systems (Povirk *et al.*, 1994). Thus, an alkaline elution study of nitrogen mustard (mechlorethamine) and melphalan showed that melphalan-induced crosslinks were much more slowly repaired, with a half-life of at least 24 h (Ross *et al.*, 1978).

The interstrand cross-link repair involves several steps such as "unhooking" step, which reflects excision of one of the crosslinked bases by a glycosylase or endonuclease. Complete crosslink repair requires subsequent removal of the adducted base in the opposite strand, as well as repair patch synthesis and ligation in both strands, a task made much more difficult by the lack of the intact template on either strand. The mechanism of crosslink repair in mammalian cells is not well defined. Most of what is known about the biochemistry of crosslinks repair has been inferred from the behavior of certain DNA repair-deficient mutants in *E. coli*, yeast and mammalian cells.

E. coli 3-methyladenine glycosylase II, the product of the *alkA* gene, was shown to release guanine-N7 adducts induced by mechlorethamine (Mattes *et al.*, 1993). The fact that *alkA* mutants are not particularly mechlorethamine-sensitive (Fram *et al.*, 1986) suggests that 3-methyladenine DNA glycosylase II does not play a critical role in crosslink removal.

uvrA mutants of *E. coli* are extremely sensitive to mechlorethamine and other interstrand crosslinking agents (Fram *et al.*, 1986) suggesting that repair of most inter-strand crosslinks requires participation of the UvrABC excision repair complex. In the

case of psoralen plus UV-A light (PUVA), such a crosslink repair deficiency has been demonstrated (Cole *et al.*, 1976). In *recA* cells, which are also profoundly mechlorethamine-sensitive (Fram *et al.*, 1986), incisions are made at PUVA crosslink sites, but are not rejoined suggesting that the completion of crosslink repair in both strands requires recombination (Sinden *et al.*, 1978). In addition, repair of interstrand crosslinks in *E. coli* may also occur by an Uvr independent activity (Van Houten 1990).

Several yeast strains with mutations at different loci have been isolated for sensitivity to PUVA which are hypersensitive to interstrand crosslinking agents. The *pso2* mutant is similar to *recA* mutant of *E. coli* in that it is thought to be blocked in interstrand crosslink repair at a step following the initial incision, since single- and double-strand breaks accumulate in PUVA-treated *pso2* cells but are not rejoined (Magana-Schwencke *et al.*, 1982). However, in contrast to *recA*, *pso2* mutants do not show any defect in recombinational repair of double-strand breaks. Interestingly, while yeast *rad52* mutant, which is X-ray sensitive and defective in recombinational repair of double-strand breaks, has a normal sensitivity to mechlorethamine (Siede *et al.*, 1992), the combination of *pso2* with *rad52* mutation results in extreme mechlorethamine sensitivity. Other yeast mutants such as *rad6* (error-prone repair pathway), *rad3* (excision repair), *pso3* (recombination) have been found to confer sensitivity to the crosslinking agents suggesting that there are multiple pathways for the repair of lethal mustard-induced damage (Povirk *et al.*, 1994).

In rodent cells, there are ten known complementation groups of UV-sensitive, excision repair-deficient mutants and two of which show 30- to 90-fold hypersensitivity to mechlorethamine, PUVA, and other crosslinking agents (Hoy *et al.*, 1985). The human repair gene *ERCC-1* complement one of the rodent groups and has homology to the yeast

excision repair gene *RAD10* (van Duin *et al.*, 1986). Paradoxically, overexpression of *ERCC-1* increases the sensitivity of normal CHO cells to melphalan (Bramson *et al.*, 1993).

Surprisingly, excision repair-deficient human xeroderma pigmentosum (XP) cell lines are not particularly sensitive to crosslinking agents (Cleaver *et al.*, 1980), raising the possibility that crosslink repair may be initiated by a glycosylase rather than by an excision repair complex (Cleaver, 1971). In any case, the excision repair system defined by the XP defect, even though it is regarded as being analogous to the UvrABC system of *E. coli*, does not appear to play a major role in repair of interstrand crosslinks induced by nitrogen mustards or other agents. Cells derived from Fanconi's anemia (FA) patients are hypersensitive to melphalan and other bifunctional alkylators (Ishida *et al.*, 1982), but are neither UV-sensitive nor excision repair-deficient. These results suggest a specific defect in interstrand crosslink repair, although the biochemical evidence for such a defect is controversial (Strathdee *et al.*, 1994).

3.4 Resistance.

There appeared to be no evidence that metabolic activation is required for cytotoxic action of melphalan in patients (Tatersall *et al.*, 1978). The same products were found in DNA isolated from alkylated cultured human tumor cells as from chemical alkylation (Osborne *et al.*, 1995 (1), Osborne *et al.*, 1995 (2)).

Melphalan like other alkylating agents is detoxified by conjugation with glutathione (GHS) by GSH transferases. Three glutathione adducts of melphalan were identified in reactions catalyzed by rabbit or human liver microsomal GSH transferases (Hamilton *et al.*, 1985). Frankfurt *et al.* (1991) have shown that the decreased response of melphalan-

sensitive cells in cocultures with resistant cells in common media without direct contact was induced by contact transfer of GSH from GSH-rich resistant cells to sensitive cells. Intercellular transfer of drug resistance was demonstrated by analysis of DNA damage and confirmed by colony formation assay. Transfer of drug resistance was prevented by phorbol ester that is known to inhibit metabolic cooperation via cell junctions.

DNA topoisomerase IIα has been associated with alkylating agent resistance including mechlorethamine, which may involve increased binding of topoisomerase IIα to alkylating agent-damaged DNA (Eder *et al.*, 1995).

Resistance to alkylating drugs can result from various sources that prevent the drugs reaching their target. Melphalan was synthesized with the hope that it could be actively transported into tumor cells as an amino-acid derivative (Bergel *et al.*, 1954), and this was later detected (Goldenberg *et al.*, 1977). Clearly, cells lacking the appropriate active transport would acquire resistance.

Bramson *et al.*(1995) extensively studied the nitrogen mustard drug resistant B-cell chronic lymphocytic leukemia (B-CLL) as an *in vivo* model for crosslinking agent resistance. They found that lymphocytes of patients with nitrogen mustard drug resistant B-cell chronic leukemia were resistant to melphalan compared to lymphocytes of untreated B-CLL patients (Bramson *et al.*, 1995). This resistance was not dependent on kinetic parameters of melphalan transport or steady-state accumulation level, or on the level of dihydroxy metabolite of melphalan. Rather, the percentage of DNA interstrand crosslinks was significantly decreased in lymphocytes from treated-resistant patients

compared to that of untreated B-CLL patients' lymphocytes due to an enhanced capacity to remove crosslinks (Bramson *et al.*, 1995).

Two well-defined repair pathways have been implicated in removal of nitrogen mustard crosslinks: base pair excision repair (BER) and nucleotide excision repair (NER). There was no increased expression of *ERCC-1* or *ERCC-2* (central members of the NER pathway) in the resistant lymphocytes, but a 1.7-fold increase in the activity of alkyl-N-purine DNA glycosylase (ANPG) (a key protein in BER) has been demonstrated in the extracts from resistant patients' lymphocytes (Bramson *et al.*, 1995). However, overexpression of neither of these three genes failed to increase resistance of CHO cells to nitrogen mustards (Bramson *et al.*, 1995). Thus, it became unclear which DNA repair system underlies the nitrogen mustard resistance in B-CLL leukemia.

These results were confirmed by further studies which revealed that resistant lymphocytes displayed strong cross resistance to mitomycin C and less so to cis-platin, but not to UV (classically repaired by NER) or methyl methanesulfonate (MMS) (repaired solely by BER) (Bramson *et al.*, 1995). As both intra- and interstrand crosslinks are implicated in the cytotoxicity of cisplatin (Szymkowski *et al.*, 1992), and only inter-strand crosslinks have been implicated in the cytotoxicity of nitrogen mustards and mitomycin C (Zwelling *et al.*, 1981, Hansson *et al.*, 1987), the partial resistant of lymphocytes to cis-platin may reflect increased repair of intrastrand crosslinks, such as in the case of UV light, but not the interstrand crosslinks. Therefore, nitrogen mustard resistance in B-CLL correlates with resistance to other interstrand crosslinking agents. This phenotype resembles the *E. coli* strain that is resistant to psoralen crosslinks and cross-resistant to nitrogen mustard and mitomycin C, but not to UV or MMS (Holland *et*

al., 1991). The pattern of hypersensitivity to psoralen crosslinks in other *E. coli* mutants suggests that genes involved in recombination are required for the repair of interstrand crosslinks (Holland *et al.*, 1991). Thus, resistance to nitrogen mustards in B-CLL is associated with enhanced repair of interstrand crosslinks that may involve a recombination dependent system.

At a given level of DNA alkylation and its repair in both sensitive and resistant cell lines, the difference in clonogenic survival can be ascribed to a late stage of the cytotoxic process that occurs after initial DNA damage and the consequent block of cell division, such as induction of apoptosis. Resistance to cytotoxic action of nitrogen mustards and other alkylation agents has been shown to result from inhibition of apoptosis in murine lymphocytic cells (Walton *et al.*, 1993) and in human myeloid leukaemia cells (Kataoka *et al.*, 1994).

3.5 Pharmacology and pharmacokinetics.

Melphalan is a direct-acting alkylating agent that is excreted unchanged in the urine and hydrolyzed non-enzymatically in the body to form monohydroxylethyl and bishydroxylethyl products. Some enzymatic attack also occurs to produce mono-and dihydroxy metabolites (Ehrsson *et al.*, 1989). It appears that the reaction products are of little toxicological importance and that the action of the drug can be associated with the parent compound. Oral bioavailability of melphalan averages 65% over the dose range of 5-20 mg, and the plasma pharmacokinetics are linear (Ehrsson *et al.*, 1989).

3.6 DNA adducts in patients under melphalan chemotherapy.

Determination of the extent of DNA alkylation in melphalan-treated tumors can serve as indicator of therapeutic effect. Immunoassay based on an antibody raised against

alkylated DNA has been used for this purpose (Tilby *et al.*, 1987). This method gave a linear response *vs.* dose down to the level encounted in patient-derived DNA samples. The immunoreactive groups on alkylated DNA are the mono-7-alkylated guanine and the crosslinked guanine, as well as a minor alkylation product in which N-7 of guanine is linked to a phosphate group. Adenine groups are not immunoreactive and crosslinked nucleotides are rather more immonoreactive than the mono-adducts.

Studies were done on peripheral blood mononuclear cells as well as on tumor cells in multiple myeloma patients receiving intravenous infusions of melphalan (Tilby *et al.*, 1993). It was shown that in peripheral blood the extent of alkylation of DNA was linear with dose reaching an average of about 8 μmol melphalan/mol DNA-P at a dose of 200 mg/m^2 1 h after treatment. There was some degree of variability between individuals and one patient among 13 studied had about double the average amount of adducts, which was correlated with a uniquely high level of toxic side effects experienced by this patient (Tilby *et al.*, 1993). Interestingly, a much higher level of adducts (27 μmol/mol DNA-P, or around 300, 000 molecules of melphalan per genomic diploid content of DNA of $1.1x10^{10}$ mol DNA-P) was found in patient whose cells derived mainly from a plasma cell tumor and resulted from a dose of 140 mg/m^2 (Tilby *et al.*, 1993). The time course of DNA alkylation showed that most of the reaction occurred within 1h of drug administration and remained much the same for at least 24 h. There was a loss of about 40% of adducts in some cases. The same kinetics was seen *in vitro* with human lymphoblastoid cells (Tilby *et al.*, 1993).

By comparing the extent of DNA alkylation found in patients with determination of myeloma cell survival in culture in relation to a given melphalan dose, it was determined that the chemotherapy would be expected to reduce survival in plasma cells to less than 10^{-4} or a cell kill of the order of 4 logs (Lawley *et al.*, 1996), assuming that the myeloma cells have the same level of alkylation per unit dose as lymphoblastoid cells in culture (14.5 μmol/mol DNA-P per unit dose of 1 μg/ml or 0.33 μM melphalan) (Tilby *et al.*, 1993). This data indicate that the extent of DNA alkylation achievable in patients does approach the desired therapeutic levels. This may well reflect the *in vitro* observations that myeloma cells are highly sensitive to melphalan (mean lethal dose D_o of 0.18 μg/ml) (Millar *et al.*, 1987), and that melphalan is actively transported into human malignant cells (Millar *et al.*, 1989). However, the paradox remains that human bone marrow *in vitro* is also highly sensitive to the cytotoxic action of melphalan with D_o of as low as 0.04 μg/ml (Robinson *et al.*, 1985).

3.7 Mutagenicity.

Early studies showed that melphalan was only marginally active in causing mutations that result in streptomycin dependence in bacteria (Szybalski *et al.*, 1958) and moderately mutagenic in the Ames tester strain TA1535, which detects base substitutions at GC base pairs (Benedict *et al.*, 1977). Unexpectedly, melphalan was found to be about 3-fold more mutagenic in this system in the presence of microsomes. It was suggested that the potentiating effect of microsomes on melphalan mutagenesis might be a result of conversion of the drug to a half-mustard, a less toxic compound (Povirk *et al.*, 1994). The relative impotency of melphalan and other aromatic mustards in Ames test strains

may be related to toxicity due to the excision repair deficiency of most of the Ames strains (Povirk *et al.*, 1994). Support for this is the observation that chlorambucil-induced reversions of the TA102 strain, which is excision-repair proficient and which is diagnostic for small deletions and several types of base substitutions, were predominantly AT>TA transversions (Yamada *et al.*, 1992).

In *Saccharomyces cerevisiae*, both mechlorethamine and melphalan efficiently reverted certain ochre (UAA) nonsense mutations indicating that these mutations were substitution at AT base pairs (Ruhland *et al.*, 1978). The much lower mutagenic potency of half-mustard analogue of mechlorethamine in the reversion assays strongly suggests that bifunctional lesions were responsible for most mechlorethamine mutagenesis in yeast. Various mustards have been shown to efficiently induce mitotic recombination and/or gene conversion in *S. cerevisiae* (Zimmerman *et al.*, 1984).

The spectrum of mutations induced by mechlorethamine in the SUP4-o gene in yeast showed diverse mixture of base substitutions occurring primarily but not exclusively at GC base pairs (Kunz *et al.*, 1989). Although most of the mutational hotspots in the gene were potential sites of interstrand (GNC) and intrastrand (GG) crosslinks, a substantial fraction of the substitutions (40%) occurred at sites where only monoadducts could be formed. For GC-AT transitions, there appeared to be a slight strand bias in favor of sites where guanine is on the transcribed strand. Such strand bias is produced by the less rapid repair of DNA damage on the non-transcribed strand (Bohr *et al.*, 1994). A *rad52* mutation (recombination repair of double-strand breaks) reduced mutagenesis two-fold and eliminated the strand bias in this system, and a *rad1* mutation (excision repair) abolished mutagenesis (Kunz *et al.*, 1989). While the latter result would be consistent

with the interstrand crosslink as the mutagenic lesion, the sequencing data indicate that only about a third of the substitutions accrued at potential interstrand crosslink sites.

Wang *et al.* (1990, 1991, 1994) carried out extensive studies on mutagenesis by various nitrogen mustards in the SV40-based shuttle vector pZ189. The plasmid was treated with the drug *in vitro*, transfected into human 239 embryonic kidney cells, allowed to replicate, and rescued in indicator bacteria; mutations in the *supF* gene of the vector were then screened and sequenced. These studies revealed that the aromatic mustards melphalan and chlorambucil, but not the nonaromatic analogue mechlorethamine, induced mutations predominantly at AT base pairs, with AT>TA transversions being most frequent. This specificity of mutations was explained to result from the thermolabile adenine-N3 adducts, since there was a correlation between adenine adducts sites and the mutation sites, and the minor groove ligand distamycin known to suppress the formation of adenine adducts also inhibited the chlorambucil-induced substitutions at AT base pairs. Induced mutations at GC base pairs occurred at sites of two or more adjacent Gs and none of the melphalan-induced mutations occurred at the potential interstrand crosslink (GNC) sites.

Melphalan-induced spectrum at the hemizygous *aprt* locus of CHO-D422 hamster cells was quite different from the spectrum in the SV40-based shuttle vector (Austin *et al.*, 1991). Very few AT>TA transversions were induced (<10%). The spectra dominated with a variety of base substitutions with GC>TA transversions being most frequent. Such mutation is typical of agents which produce bulky guanine adducts. According to the so called "A rule" replication of chemically modified DNA bases will tend to result in the insertion of adenine in the newly replicated DNA, opposite the lesion (Strauss *et al.*,

1991, Strauss *et al.*, 1992). This rule derives from a tendency for DNA polymerase to act as if such damage was the major effect of UV radiation involving thymine. Thus, bulky adducts, including crosslinks, are expected to cause GC>TA transversions more frequently, if indeed the adducts are formed at G residues. Guanine at GGC and GGCC sequences were particularly frequent mutation sites in CHO- D422 cells (Austin *et al.*, 1991). However, the mutations could not be correlated with the sites of adducts formation. The examination of guanine-N7 adducts in a GGC oligonucleotide duplex revealed the formation of interstrand crosslinks at the 5'G in the sequence, but neither interstrand nor intrastrand crosslinks were detected at the 3'G, which was the more frequent mutation site in the *aprt* gene (Bauer *et al.*, 1994). Therefore, the data gives little indication on which adducts might be involved in melphalan-induced mutagenesis.

Deletions were induced in both CHO cells and in pZ189 vector, and processing of DNA breaks after the spontaneous depurination and strand cleavage at sites of adducts might have produced such mutations. From the data above it appears that both monofunctional and bifunctional adducts, and adducts at both adenine and guanine contribute significantly to the mustard-induced mutagenesis, and that the relevant importance of various specific adducts varies markedly between assay systems.

Mustards produced diverse mutations in various other mammalian cell lines. Mechlorethamine-induced spectra in the *hprt* gene of human lymphoblastoid line dominated with partial deletions clustered within a small region of exon 6 (Henner *et al.*, 1991). The majority of chlorambucil-induced mutations in the *hprt* gene in hamster V79 cells were not large deletions as demonstrated by the normal-length PCR products for the *hprt* gene (Speit *et al.*, 1992). Melphalan and other alkylating agents were mutagenic at

the *hprt* locus of human lymphoblastoid cells (Sanderson *et al.*, 1991). Folate deficiency was shown to act synergistically with alkylating agents to increase somatic mutation in CHO cells (Branda *et al.*, 1993 (2)).

Melphalan was shown to induce specific locus mutations in germ cells and spermatogonial stem cells of male mice (Generoso *et al.*, 1995), dominant lethal mutations and heritable translocations in male mice and strong reproductive effects in female mice (Russel *et al.*, 1992 (1,2)). Molecular analysis of melphalan and chlorambucil-induced recessive visible germline mutations in the mouse by Southern blotting has shown that these mutations are predominantly large, multilocus deletions (Rinchik *et al.*, 1990).

Dose-dependent increase in the *hprt* mutant frequency was detected in spleen lymphocytes of cyclophosphamide-treated rats (Aidoo *et al.*, 1991) and mechlorethamine, chlorambucil, and cyclophosphamide all gave positive results in the *lacI*/lacZ-based transgenic mouse mutagenesis assays (Myhr 1993, Kohler *et al.*, 1991).

3.8 Chromosomal effects.

All nitogen mustards including melphalan are potent clastogens that induce a wide range of chromosomal aberrations as well as SCE (Povirk *et al.*, 1994). Results of banding studies (Reeves *et al.*, 1974, Mamuris *et al.*, 1989 (1)) showed the nonrandom nature of mustard-induced damage in human lymphocytes in that the breakpoints of most translocations and deletions were localized to R-bands or G-light-band regions. These regions are known to be GC-rich, to contain a preponderance of constitutively expressed genes and to be less condensed in interphase than G-dark bands (Manuelidis *et al.*, 1990).

Each of these properties could contribute to the apparent greater susceptibility of these regions to mustard-induced clastogenesis.

Mamuris *et al.* (1989 (2)) studied the evolution of melphalan-induced chromosome abnormalities in human lymphocytes. Melphalan is considered a typical S-dependent mutagen, thus principally inducing monostrand lesions. This was confirmed by the fact that at first mitosis following mutagenesis two thirds of observed lesions were chromatid breaks or exchanges. The strongest hot spot for the breaks was 9q1 which is also a frequent site of spontaneous breaks. Additionally, there was a marginal excess of breaks in chromosomes 5,7,11, and 17 where aberrations are frequently seen in leukemic cells of patients with mustard-associated leukemias. The specificity of chromosomes 1,5,7,11 and 19 involved in rearrangements was preserved in 96 h and 120 h cultures, however, the preferential involvement of R-band rich segments became less obvious, decreasing from 72 h cultures. Also, the distribution of aberrations shifted over the next several generations in favor of chromosome-type aberrations including mostly unbalanced rearrangements such as deletion, dicentrics and complex rearrangements. The deletions increased from 72 to 96 h indicating that many breaks and gaps result in deletions. However, deletions decreased from 92 to 120 h, which may be due to the selective disadvantage of these cells. The striking exception to that was the observation that for chromosomes 5 and 20 a significant excess of deletions was observed 96 h and 120 h after mutagenesis while there was a significant decrease in deletions in chromosome 9. This indicates that del (5) and del (20) are poorly eliminated by selection and this finding may be related to the fact that del (5) and del (20) are frequently observed in preleukemic syndromes after chemotherapy with alkylating agents. However, this data is not in

agreement with results obtained *in vivo* in melphalan-treated patients where the only aberrations showing persistent elevation (1-7 years post-treatment) were translocations (Lambert *et al.*, 1984).

The mechanism that leads to chromosomal aberrations and SCE by melphalan is still uncertain. The principle that bifunctional alkylators are much more effective clastogens than monofunctional alkylators suggests that aberrations result largely from bifunctional melphalan-induced lesions, such as intra- and interstrand, and DNA-protein crosslinks. The model proposed for the aberration formation postulates that DNA breakage is required for the resolution of replication forks arrested at sites of interstrand crosslinks (Povirk *et al.*, 1994). Such DNA breakage could lead to chromatid breaks and, through repair, to other aberrations such as translocations. This model is supported by the observation that passage of cells through S-phase is required for the aberrations to occur (Stevenson *et al.*, 1973), and by the fact that cells from FA patients, that are believed to have a defect in interstrand crosslink repair, are acutely hypersensitive to mechlorethamine (Sasake *et al.*, 1973).

3.9 Carcinogenicity.

The carcinogenic potential of nitrogen mustard was demonstrated in mice (Boyand *et al.*, 1949) and mustard gas was identified as a cause of lung cancer in man (Wada *et al.*, 1968). Melphalan and other chemotherapeutic alkylating agents are recognized as human carcinogens (IARC 1987). Carcinogenic potency of these agents in rodents and humans correlates better with the total exposure to active chemicals rather than with the life-time average concentration (Dedrick *et al.*, 1992).

The risk of developing sAML (including MDS) in multiple myeloma patients treated with alkylating agents has been estimated to be 17% (actuarial risk at 4 years) (14 sAML cases developed in 364 MM patients, RR=264) and to be 10% (actuarial risk at 10 years) (Rodjer *et al.*, 1990). The highest risks of sAML in ovarian cancer patients were found after previous chemotherapy including melphalan and chlorambucil (RR range from 22 to 100), especially when given in large doses over prolonged periods (Curtis *et al.*, 1984). A significant excess risk of developing a new primary solid tumor was detected in ovarian carcinoma patients treated with melphalan (RR=3) (Einhorn *et al.*, 1988). In patients with primary systemic amyloidosis treated with a median melphalan dose of 1764 mg the actuarial risk of sAML and MDS was 21% at 3.5 years (Gertz *et al.*, 1990). Treatment with other alkylating agents have been associated with an increased leukemia risks in patients with primary Hodgkin's disease, non-Hodgkin's lymphoma, breast cancer, lung cancer and other neoplastic and nonneoplastic conditions (Curtis *et al.*, 1984). On the other hand, the 10-year cumulative incidence of myeloid leukemia (1.3%) was low in children with primary Hodgkin's disease and non-Hodgkin's lymphoma indicating that increasing age predispose to development of sAML in these patients (Pui *et al.*, 1990).

3.10 Genetic damage and secondary leukemia with -5/5q-, -7/7q- deletions after melphalan chemotherapy.

Several studies showed that melphalan chemotherapy is associated with genotoxic damage that can be long lasting. Patients receiving melphalan chemotherapy were shown to have transient two- to three-fold increases in the SCE frequency in peripheral lymphocytes which declined to near pre-treatment levels over a four-week period (Lambert *et al.*, 1986, Popp *et al.*, 1992). However, it can take months for SCE frequency

to fall to the level of untreated controls (Popp *et al.*, 1992, Raposa *et al.*, 1987). *In vitro* studies showed that SCE frequency induced by melphalan reduced slowly in resting Go lymphocytes and considerably faster in mitogen-stimulated G1 cells (Lambert *et al.*, 1986). At the same time, the frequency of chromosomal aberrations in ovarian cancer patients was increased for up to 10 years after melphalan therapy (Lambert *et al.*, 1993).

The elevated DNA crosslinking have been detected in patients after melphalan chemotherapy before the beginning of a subsequent therapy cycle, while the crosslinking frequency did not change appreciably during the cycle (Popp *et al.*, 1992). The highest frequency of DNA crosslinking was reached after several hours of therapy and was repaired very slowly (Popp *et al.*, 1992).

Banding studies in patients treated with melphalan have shown the significant increase in chromosome-type lesions in patients' lymphocytes with chromosomes 5, 7, 9 and 11 preferentially affected (Mamuris et al., 1989 (3)). This coincided with the lymphocyte abnormalities observed in the same category of patients who developed sAML (chromosomes 5, 7, 11, 17) (Mamuris *et al.*, 1990) and after *in vitro* treatment of normal lymphocytes by melphalan (Mamuris et al., 1989 (2)). The frequent breakage of chromosomes 5, 7, 11, and 17 and of R bands in general, which are known to be G-C rich, may result from the preferential DNA-adduct formation at guanine bases. The chromosomes 1, 5, 7, 11, and 17 are among most frequently rearranged or deleted in leukemic cells of sAML after cytotoxic chemotherapy involving alkylating agents (Braekeller 1986). This similarity rises the possibility that chromosomal abnormalities in sAML are induced by the treatment and that melphalan could play an important role in the pathogenesis of sAML. However, it remains unlikely that the deficiencies observed

in leukemic clones were directly induced at the time of treatment. It was proposed that treatment of primary cancers induces nonrandom mutations of recessive cancer genes (such as tumor-suppressors) located on the chromosomes most often affected by the rearrangements. Consequent various rearrangements, including deletions of the homologous normal counterparts, unmask mutated recessive genes and lead to leukemogenic process (Mamuris et al., 1989 (3)).

Regions affected most often by deletions in sAML and myelodysplasia after alkylating agent (including single agent melphalan) chemotherapy are deletions or loss of the long arms of chromosomes 5 and 7 (Pedersen-Bjergaard et al., 1994, Pedersen-Bjergaard et al., 1995). Attempts have been made to identify the putative tumor suppressor genes located at the most commonly deleted regions. The 5q21-33 region involved in 5q deletions in myeloid and lymphoid leukemias has been extensively studied. The smallest commonly deleted region on 5q has been identified. It consists of band 5q31, where several genes are located encoding growth factors, hormone receptors, and proteins involved in signal transduction or transcriptional regulation (Benedict *et al.*, 1977). It was suggested that the loss of the tumor suppressor releases the malignancy, interleukin losses determine the hematology, and deletion of other genes affects survival (Povirk *et al.*, 1994). Two candidate tumor suppressor genes identified in this region are EGR1 and IRF-1 (Povirk *et al.*, 1994, Yamada *et al.*, 1992).

The breakpoint region 7q22-34 involved in deletions has been identified, and the gene on 7q31 has been shown to suppress the expansion of the 7q- clone and delay cytogenetic progression (Kunz *et al.*, 1989).

4. Multiple myeloma (MM).

4.1 Description, incidence, risk factors and treatment.

Myeloma is a prototype of a group of conditions known as plasma cell neoplasms (Oken 1991). Plasma cell neoplasms are a group of related disorders associated with proliferation and accumulation of immunoglobulin-secreting cells that are derived from the B-cell series of immunocytes. Monoclonal components occur in both the malignant plasma cell disorders (such as multiple myeloma) as well as in the clinically unclear or idiopathic benign, premalignant, or early malignant disorders.

Multiple myeloma is a neoplasm of malignant plasma cells invading bone and bone marrow, causing widespread skeletal destruction, bone marrow failure and problems related to quantitatively abnormal serum or urinary monoclonal protein (M proteins) (Markman 1994). The basic immunoglobulin unit comprises two identical heavy chains linked to two identical light chains. The heavy chain is either γ, α, μ, δ, or ε corresponding to IgG, IgA, IgM, IgD and IgE respectively. The light chains exist as either κ or λ subtypes. Serum M protein is a monoclonal whole immunoglobulin and therefore possesses only one heavy chain type and one light chain type. Urine M protein consists of three light chains or, the case of some heavy chain diseases, three heavy chain fragments of single specificity. The monoclonal immunoglobulins secreted in malignant plasma cell disorders are similar to normal homogenous antibodies, but in most instances the antigen they bind is unknown. Multiple myeloma is often diagnosed when a monoclonal protein is found in the serum or urine or both. The diagnosis also requires histologic documentation by the demonstration of increased numbers (usually >10%) of abnormal, atypical, or immature plasma cells in the bone marrow.

The annual incidence of multiple myeloma is 3/100,000 population with a peak occurring between ages 60 and 70. Multiple myeloma accounts for approximately 1% of all malignant neoplasms in the US and affects blacks twice as often as whites.

Most patients with multiple myeloma require chemotherapy for their disease soon after diagnosis. Patients who do not undergo chemotherapy or who make up the 40% of patients for whom chemotherapy is not effective can expect to survive less than a year. Responders have a median survival of only 3 to 4 years. The melphalan-and-prednisone regimen improves median survival from 7 months to 3 years in the 50% to 60% of patients who respond. Cure is exceedingly rare.

Possible risk factors for multiple myeloma include radiation exposure (people who entered Hiroshima city within 3 days after the blast had a nearly 60% greater risk of dying of myeloma than those not exposed), preexisting medical condition (chronic antigenic stimulation through bacterial infections, diabetes mellitus, medical implants, allergies and related disorders in some studies), occupational exposure (radiologists, farmers), medications (propoxyphene, possible association with phenytoin, phenobarbital, diazepam, propranolol, ibuprofen, diet drugs, stimulants and lazatives, as well as erythromycin, chlorpheniramine, gentamicin, sulfamethoxazole and terpin hydrate), and family history.

There are two major treatment regimens of the standard induction chemotherapy, which have similar response rates (72% and 51%) and median survival (26% and 19% of 5-year survival). More intense regimen includes vincristine, carmustine, melphalan, cyclophosphamide and prednisone, while moderate therapy, recommended for patients over 70 years of age who have poor performance status, includes cycles of melphalan and

prednisone. Because of the similarity in median survival between the two induction regimens the later regimen can also be considered for some patients not in a high-risk group if a less aggressive approach is required.

Objective response to chemotherapy has a median duration of about 2 years. Eventually, virtually all patients develop recurrent or refractory disease. For truly resistant myeloma, which clearly progresses during initial therapy, high-dose or pulsed glucocorticoids appear to be the best treatment, with an expected response rate of 40%. For patients who have a relapse during therapy or within 6 months afterwards, the combination of vincristine, doxorubicin, and dexamethasone is one of the most effective salvage therapies, resulting in approximately a 75% response rate. Other maintenance regimens include high-dose cyclophosphamide, whole body irradiation with bone marrow transplantation, IL-2 and interferon alpha therapy.

4.2 Clonal chromosomal abnormalities.

Cytogenetic studies on bone marrow samples identified abnormalities in around 50% of MM patients (32% and 51% in different studies) (Sawyer *et al.*, 1995, Lai *et al.*, 1995). Treated patients have higher number of abnormalities compared to untreated patients tested at diagnosis (35% *vs.* 18% and 71% *vs.* 47%). The abnormal karyotypes generally show numerous numerical and structural aberrations and in some patients multiple abnormal clones. Hyperdiploidy was observed in 30% of patients and gains predominantly involved chromosomes 3,5,7,9,11,15,19 and 21. Hypodiploidy was observed in 15% of patients and frequent loss affected chromosome 13 including interstitial deletions involving band 13q14 (43% in one study). The most frequent structural aberrations were those of chromosome 1 (48% and 15% in different studies).

The single most frequent chromosome breakpoint involved band 14q32 (33%) and 14q+ chromosome with t(11;14)(q13;q32) and t(8;14)(q24;q32). Translocation t(8;14)(q24;q32) has been associated with IgA myeloma protein. The same translocation has been associated with 80%-90% of all Burkitt's lymphoma cases where *c-myc* oncogene on 8q24 is juxtaposed to immunoglobulin heavy-chain (*IgH*) genes on 14q32 (Solomon *et al.*, 1991). Translocation t(11;14)(q13;q32) juxtaposes *IgH* genes to the *bcl-1* (PRAD1) oncogene, a translocation often found in B-CLL and diffuse B-cell lymphoma (Solomon *et al.*, 1991).

5. Small cell lung cancer (SCLC).

5.1 Description, incidence, risk factors and treatment.

Lung cancer is the leading cause of cancer-related deaths for both men and women in Canada with estimated 1997 incidence accounting for 17.8% and 12.9% of all cancers and 32.5% and 22.4% of all cancer-related deaths in males and females respectively (Canadian Cancer Statistics). Cigarette smoking is a major risk factor and accounts for 85% of all lung cancer cases, however, only 10-15% of heavy smokers ultimately develop lung cancer. This indicates the effect of inter-individual variation in cancer susceptibility to similar carcinogen exposure, which may depend on genetic polymorphism in carcinogen metabolism and DNA repair. The candidate biomarkers for such polymophisms have been identified and include cytochrome p450 enzymes, glutathione S transferase and N-acetyltransferase (Raunio *et al.*, 1995). DNA adduct profile in peripheral lymphocytes representing a sum of carcinogen activation/detoxification reactions as well as DNA repair are potential susceptibility markers. Lung cancer patients tend to have a higher level of benzo[a]pyrene diolepoxide

(BPDE)-induced DNA adducts than cancer-free control did (Li *et al.*, 1996). Other risk factors include exposure to high doses of ionizing radiation, radon, asbestos, other occupational agents such as mustard gas, chloromethyl ethers, chromium, nickel and inorganic arsenic and air pollutants such as polycyclic aromatic hydrocarbons (PAHs).

Epidemiological studies have suggested that susceptibility to lung cancer may have a Mendelian inheritance (Gazdar 1992). Candidate inheritable traits include genes for carcinogen metabolism, such as p450 enzymes, xenobiotic metabolism, and tumor suppressor genes. There is an increased risk of developing a lung cancer, particularly SCLC, in familial retinoblastoma patients and in Li-Fraumeni syndrome patients.

SCLC accounts for 25% of all cases of lung cancer. This tumor has many clinical and pathological features that distinguish it from the other major types of lung cancer such as squamous cell carcinoma, adenocarcinoma, and large-cell carcinoma, collectively referred to as NSCLC. In contrast to NSCLC, where surgical resection offers the only hope for cure and/or long-term survival, SCLC is sensitive to chemotherapy and the usual treatment consists of combination chemotherapy including vincristine, doxorubicin and cyclophosphamide or cisplatin and etoposide with or without radiation. Chemotherapy achieves response rates of up to 90% with median survival ranging from 8 to 18 months compared to 6-12 weeks with supportive care only (Seifter *et al.*, 1988). Single-agent etoposide chemotherapy demonstrated that this agent is among the most effective in the management of SCLC (Carney *et al.*, 1992). Oral etoposide is preferred treatment regimen in the treatment of unfit or elderly patients because such patients have a poorer response to overall systemic chemotherapy and a shorter survival time than do their younger counterparts due to the toxicity associated with systemic chemotherapy. It was

shown that among elderly persons single-drug etoposide chemotherapy is well tolerated, has a response rate comparable with that seen with combination chemotherapy (76% *vs.* 88%) and comparable median survival (38 wks *vs.* 42 wks).

Despite initial responses to chemotherapy the majority of patients eventually succumb to their disease, and the major reason for treatment failure is the acquisition of resistance to multiple chemotherapeutic agents. The multidrug resistance has been associated with overexpression of transport protein P-glycoprotein (MDR1), multi-drug resistance protein (MRP) (Jain *et al.*, 1996) and with the reduction in the quantity and/or function of topoisomerase IIα (Jain *et al.*, 1996).

5.2 Chromosomal aberrations and mutations in SCLC.

Many clonal cytogenetic abnormalities are found in lung cancer with lesions in known and putative tumor suppressor genes. Loss of 3p heterozygosity occurs in 95% of SCLCs and in over 50% of NSCLC (reviewed in Gazdar 1992). Several separate regions are targeted including 3p21, 3p24-25, and 3p14, as well as sites on the 3q arm. These findings suggest involvement of several candidate tumor suppressor genes, including the Von Hippel-Lindau disease gene, *raf* proto-oncogene, β receptor for retinoic acid, zinc-finger-containing genes, and tyrosine phosphatases. Loss of heterozygocity (LOH) occurs in 11p, 13q (retinoblastoma susceptibility gene RB-1), and 17p (p53 gene). In SCLC, the 13q and 17p exhibit LOH in over 90% of cases, however, only in 20-50% of cases in NSCLC. LOH at another loci 9p occurs at the earliest stage in the pathogenesis of NSCLC.

In SCLC p53 mutations are found in 61% of cases and in almost all cases the normal allele was lost (Lohmann *et al.*, 1993). NSCLC has less p53 mutations (34.5% of cases) (Sarkar *et al.*, 1995). Mutations at the p53 gene in lung cancer have been associated with cigarette smoking, radon and asbestos exposure (Taylor *et al.*, 1994, Wang et al., 1995). The predominance of GC>TA transversions in the spectra (40%) reflects a possible genotoxic influence of carcinogens present in tobacco smoke. The tobacco smoke carcinogen benzo[a]pyrene diolepoxide (BPDE) was shown to induce point mutations which are mainly GC>TA transversions in human T-lymphocytes at the *hprt* gene (Lambert *et al.*, 1994). Retinoblastoma gene (*rb*) mutations are almost always present in SCLC whereas in NSCLC the incidence is much lower (20%).

The oncogenes associated with lung cancer include the *myc* and *ras* families, the *neu*, *myb*, *raf*, and *bcl-1* genes, the epidermal growth factor (EGF) receptor gene, and platelet-derived growth factor. Amplification of *myc* in a subset (10%) of SCLC is associated with prior specific cytotoxic therapy and is rare in lung cancers and cell lines from previously untreated patients. Amplification of *myc* genes in NSCLC is relatively uncommon. Mutations in *ras*, particularly K-ras gene codon 12, are found in 30% of NSCLC with adenocarcinoma having higher (57%) proportion of cases occurring at a relatively early stage. As with p53 mutations, K-ras mutational spectra is dominated by GC>TA transversions and is associated with life-time smoking (Husgafvel-Putsiainen *et al.*, 1993). However, no *ras* mutations were found in SCLCs. Patients with *ras* mutations have a significantly poor survival rate.

6. *hprt* T-cell clonal assay: history and implications.

6.1 History.

Various methods have been developed for monitoring mutation in human populations (Cole *et al.*, 1994). Blood has been the obvious tissue of choice in such studies due to the availability of samples. The genetic end points include hemoglobin variants and glycophorin A variants in erythrocytes and mutation in *hprt* (autoradiographic and clonal assays), HLA A and CD3/T-cell receptor complex in lymphocytes (Cole *et al.*, 1994). The *hprt* T-cell clonal assay is the most commonly used in human monitoring studies. It is based on the isolation of mononuclear cell fraction from peripheral blood sample and *in vitro* culture of T-lymphocytes with mitogen PHA, T-cell growth factor interleukin-2 and irradiated feeder cells in 96-well microtitre plates. Under these conditions, T-cells divide to produce clones that can be scored after approximately 14 days incubation under light microscope. The cells mutated in the *hprt* gene can be readily selected in the medium containing a toxic base analog 6-thioguanine (6TG) which can not be incorporated into DNA by a mutant HPRT enzyme (see below). As a result of the 6TG exposure, $hprt^-$ cells die but the *hprt* mutant cells survive and proliferate. The number of positive and negative wells on each plate is then determined and the cloning efficiencies (CE) in both selective and non-selective media are calculated based on the relationship:

$$P=e^{-x}.$$

It is assumed that the fraction of wells without colony growth (P) follows the Poisson distribution, where x is the number of cells/well plated (usually 1 to 10 in non-selective medium or $2x10^4$ in selective medium). CE is then calculated according to the formula:

$$CE=-\ln P/x.$$

The mutant frequency (MF) is calculated as a ratio of CE in the presence of 6TG to the CE without 6TG. The advantage of this assay is that the mutant clones can be expanded

and the nature of the mutation identified. Furthermore, the comparison of the T-cell receptor (TCR) gene rearrangements in individual clones permits the identification of mutants' clonality. Thus, the *hprt* MF as well as the spectrum of independent *hprt* mutations can be obtained.

The *hprt* T-cell clonal assay was developed by Albertini *et al.* (1982, 1985). Factors influencing measurements of MF in this assay have been investigated in great detail and modifications to the original method allowing for higher CE or greater number of clones able to expand have been proposed (Cole *et al.*, 1988, Beare *et al.*, 1993). This assay was intended to become a system for direct mutagenicity testing in human population. The *hprt* MF was investigated in normal unexposed population, in populations occupationally or accidentally exposed to known mutagens and carcinogens, in donors with cancer-prone genetic disorders or DNA-repair deficiency, in cancer patients before treatment and in cancer patients after radiotherapy and/or chemotherapy treatment (Cole *et al.*, 1994). The result of these studies was a demonstration that an increased mutation frequency at the *hprt* locus can be detected in patients with cancer-prone syndromes and in exposed populations compared to the unexposed healthy control population (Cole *et al.*, 1994). However, a great inter-individual variation and a complex or unknown nature of exposure may present difficulties in interpreting the results. Additionally, in many cases the MF did not change as a result of the potentially mutagenic treatment. Also, the *hprt* assay in itself presents a wide array of sources of variation in determining the MF. Therefore, attention should be given to all of these factors when the *hprt* T-cell clonal assay is used in human monitoring studies of genotoxic exposure.

Since its development, many laboratories have used this method for monitoring *in vivo* and *in vitro* genotoxic exposure in various systems. An extensive *hprt* database has been created which includes a list of all published *hprt* mutations with the reference to the MF, CE and system used to obtain the mutants (MutaBase Software, Durham, NC).

6.2 Spontaneous *hprt* MF in unexposed human population.

To be able to detect the genotoxic effect of exposure in humans it was first necessary to determine the "spontaneous" *hprt* MF or MF in healthy control population with no known history of mutagenic exposure at their home, work place, or by radiotherapy or chemotherapy. Various laboratories have been working on establishing the baseline *hprt* MF in human population and on understanding the factors that might affect the MF.

First of all, it was determined that there is a wide range in the "spontaneous" or "background" *hprt* MF in unexposed adults which may vary by over 2 orders of magnitude in different donors (Cole *et al.*, 1994). Given the variation between the subjects, it was calculated that from 30 to 50 subjects per subject category may be required to have a 90% chance of detecting a 1.5-fold increase in MF above the control level. A smaller sample size of around 20 per group would be required to detect a 2-fold increase (Robinson *et al.*, 1994). Also, a comparative study showed that the overall data were in general agreement between different laboratories with MF being in the same range for various population groups despite the differences in experimental techniques (differences in the PHA use or concentration, IL-2 source, source of the feeder cells, MNC density, 6TG concentration, use of alternative serum, technique and experience of individual laboratory worker) (Cole *et al.*, 1994, Robinson *et al.*, 1994). Further, it was determined that the MF per clonable cell was inversely proportional to CE (a 20%

increase in CE was associated with 20-29% reduction in MF) that was generally high (>30%). Gender had no effect on MF, age had an apparent effect, while smoking was associated with a significant increase in MF in some laboratories (by a factor of 1.5) but not in others. Differences in sample size, type of the tobacco and smoking habits as well as difficulties in their assessment may contribute to the discrepancy.

The effect of age and CE on MF has been studied by many groups and various lnMF= f (age, CE) relationships have been constructed (Cole *et al.*, 1994). It was found that in cord blood (newborns) the MF was significantly (about 10-fold) lower than in young adults (the overall mean is about 0.8×10^{-6} compared to 6×10^{-6} in adults). In adults the age-related increase in MF was estimated to be 1-5% per year. Our group took a different approach in that we partitioned the MF by subject age and determined the effect of age in each partition individually (Curry *et al.*, (submitted)). We have analyzed a total of 1438 subjects available in the literature and found that the MF (mean 0.98 ± 0.89) as well as CE (0.30 ± 0.20) were significantly lower in newborns (group 1) than in other partition groups. The mean MF significantly increased to 2.66 ± 2.60 in group 2 (age 1-15), further to 8.73 ± 5.69 in group 3 (age 16-50) and lastly to 14.28 ± 9.27 in group 4 (age 51-85). The CE, however, was independent of age in the last three groups. The MF increased rapidly ($\ln MF = -0.16 + 0.11 age$, $p<10^{-6}$) in group 2 (age 1-15), continued to increase significantly but not so rapidly ($\ln MF = 1.00 + 0.03 age$, $p<10^{-6}$) in adult population (age 16-50), and then leveled off in the older generation (age 51-85) where it was independent of age ($\ln MF = 1.82 + 0.01 age$, $p=0.046$). Consistent with the results from other

laboratories, the lnMF in our study was inversely proportional to the CE with a similar slope for all four groups.

Various reasons for such age effects on CE and MF can be suggested. The lower CE and MF in cord blood may be a reflection of the biology of the T-cells in this age and, perhaps, their limited capacity to undergo PHA/IL-2 activation into clonal expansion. The rapid increase in the MF in small children is, probably, primarily due to the clonogenic proliferation of T-cells induced by antigenic stimulation. Proliferation confers the creation of mutation and colony growth allows for the detection of mutants in a much larger population of *hprt* non-mutants. Transient increases in MF can be attributed to such factors as environmental exposure to DNA-damaging agents, infection and gradual accumulation of long-lived mutant T-cells. The leveling off of MF in elderly people (>50 years) can be explained by the fewer cell divisions (as one of the consequences of an aging immune system) and thus less opportunities for mutation to become "fixed". Other factors such as altered ability of T-cells to respond to PHA and feeder cell stimulation, less production of and responsiveness to IL-2, altered composition of T-cell subpopulations (see below) may also play a role (Chrysostomou *et al.*, 1984).

We, thus, concluded that the greatest contribution to mutation at the *hprt* gene in human T-cells occurs early in life and diminishes with age until after age 50 when the rate of detectable mutation becomes constant. We also found that the mutational spectrum, with the exception of AT> CG transversions which increase with age, did not change remarkably with age in adults. We, thus, found no evidence to support the models of aging that predict that mutation increases in the elderly. However, it is important to

emphasize that the *hprt* mutations are measured in T-cells and we might simply observe events peculiar to T-cells.

T-cell subsets have been shown to influence the *hprt* CE and MF. Human T-cells can be classified in two main subsets: helper T-cells (CD4) and cytotoxic T-cells (CD8) (see below). The proportion of T-cell subsets varies with age and is affected by smoking and genotoxic exposure. Thus, as much as 50% reduction in CD4/CD8 ratio was reported in chemotherapy and radiotherapy patients and in nurses and pharmacists exposed to antineoplastic drugs (Petrini *et al.*, 1983, Dubeau *et al.*, 1994). Albertini *et al.* (1985) reported that CD4 cells are overrepresented in the clonal assay. Dubeau *et al.* (1994) showed that CE is positively associated with the number of CD4 cells while the CE in the selective medium was not associated with the proportion of CD4 or CD8 cells. They also observed CD4/CD8 ratio of 9:1 among non-selected clones and 3:1 among selected clones suggesting the differential potential of various T-cell subsets to proliferate in selective and non-selective media (Dubeau *et al.*, 1994). This may reflect the more complex requirements for CD8 cells for *in vitro* proliferation and, therefore, their growth advantage in the selective media with thousands of other T-cells in the media. Therefore, differences or changes in T-cell subsets may be a confounding factor in accessing the CE and MF after genotoxic exposure.

Despite the general agreement of MF data obtained in different laboratories there are several sources of variation in the estimation of MF including experimental and donor variation. Freezing of cells prior to analysis did not seem to affect the MF (Cole *et al.*, 1994). Experiments with split and repeat blood samples showed that the observed MF for any given donor can vary 2-8-fold between experimental determinations with the greatest

variation being between different samples from a single donor (Cole *et al.*, 1994). The inter-individual variation was even greater (individual adult donor MF ranged from <0.5 to 111.7) (Cole *et al.*, 1994). When comparing three laboratories, the variation (σ^2) assigned to split samples ranged from 0.087 to 0.401, the variation assigned to repeat samples ranged from 0 to 0.128, and the between subject variation ranged from 0.028 to 0.158. The overall variance per subject ranged from 0.334 to 0.446 (Robinson *et al.*, 1994). The variation in repeat samples may reflect the complex nature of T-cells such as T-cell turnover, relative proportions of T-cell subsets and their clonal potential. The variation in split samples may reflect the differences in media and technique used. These observations led to the suggestion that a single estimation of MF from an individual donor can not be considered in isolation and that the statistical analysis should always be based on log (geometric) mean MF (Cole *et al.*, 1994).

Another important factor contributing to variation in MF is the ability to obtain high CE as the CE is inversely proportional to MF. It is important to insure that there is no selective disadvantage for any T-cell subsets in both selective and non-selective media. The proportion of T-cells in the MNC fraction from the blood is 60-85% and most or all of them are potentially clonable (Cole *et al.*, 1988). While some groups obtain a consistent high CE (mean 50%), the others report much lower values (10-20%) (251). Therefore, it is important to adjust for CE when comparing data sets.

6.3 Chemotherapy studies.

There have been several studies on the effect of chemotherapy on *hprt* mutation frequency in cancer patients. While having an advantage on knowing the drugs and doses

used in the treatment such studies have limitations. A wide variety of cancer types, use of multiple drugs in multiple cycles, difficulties with obtaining blood samples at particular times and varying individual reaction to treatment all make the interpretation of results particularly difficult (Cole *et al.*, 1994). It was also noted that the "highly toxic therapy is not a good model for deducing the frequency and kinetics of *in vivo* mutant expression in blood-derived cells" (Cole *et al.*, 1994).

Tates *et al.* (1994) studied 15 cancer patients (mean age 36±16 ranging 16-66) including 10 testicular carcinoma patients, and others with leiomyosarcoma, ovarian cancer, osteosarcoma, breast cancer and soft tissue sarcoma treated with several types of combination chemotherapy including such drugs as topoisomerase II inhibitors etoposide and adriamycin (dosorubicin), alkylating agents cyclophosphamide, ifosfamide and cis-platin, DNA cutting agent and free radical generator bleomycin, mitotic spindle inhibitors vinblastine and vincristine and antimetabolites methotrexate and 5-fluorouracil. The *hprt* MF was analyzed in patients' peripheral blood lymphocytes before, during and after chemotherapy. The mean MF before treatment was not different from the historical healthy donor controls ($9.1\pm3.9\times10^{-6}$ compared to $11.6\pm12.2\times10^{-6}$ in the controls). The mean MF for treatment plus post-treatment samples ($15.9\pm15.0\times10^{-6}$) was found to be significantly elevated compared to historical controls. The authors concluded that the increase could be fully ascribed to 10 patients treated with at least one of the following agents: ifosfamide, cyclophosphamide, adriamycin or 4-epi-adriamycin (group 2). However, the analysis of data showed that in a group of patients where the MF increased after treatment, the CE had either decreased or stayed the same compared to pre-treatment

values from 33.3±13.1 to 31.5±18.4, whereas in the rest of the patients, the CE increased from 37.3±17.5 before treatment to 48.1±19.8 after treatment. Because ln MF is inversely proportional to the CE, it is important to correct for CE when comparing MFs before and after treatment. However, even when the MF was corrected for fluctuations in CE, the mutagenic effect was significantly more pronounced in group 2 patients and even more so in post-treatment rather than treatment samples. The mean expression time for the induced *hprt* mutations was estimated to be 98±54 days. Based on data from two patients, the enhanced MF persisted for at least 430-490 days after the termination of treatment. In three patients with testicular carcinoma (age 28, 44 and 46) treated with etoposide and cisplatin, a significant decrease in MF was observed during chemotherapy. There were also 5 patients in whom the frequency of micronuclei increased 2-6-fold during and after treatment. No particular drugs or cumulative doses could be attributed to the elevated micronuclei frequencies in these patients.

Hirota *et al.* (1993) analyzed the *hprt* mutation following anti-cancer treatment in pediatric patients with acute leukemia. The study included 45 children with ALL (age 12.2±5.1), 13 children with AML (age 11.8±6.0) and 28 healthy age-matched (age 9.8±5.45) controls. The patients had previously received intensive chemotherapy with multiple drugs (including cyclophosphamide in ALL patients and etoposide in AML patients) and 84% of ALL and 92% of AML patients had also received radiotherapy. Patients were examined at different times after the completion of therapy (maximum 82 months) and 26 patients were examined at least twice at intervals of 6 to a maximum of 24 months. The CE in this study was consistently high for all groups of patients

(62±3.7% in controls, 56.4±7.3% in AML patients and 59.0±3.8% in ALL patients). The mean MF was $1.1\pm0.2\times10^{-6}$ in controls, $1.7\pm0.6\times10^{-6}$ in AML and $7.8\pm2.6\times10^{-6}$ in ALL patients and was significantly increased in ALL compared to AML patients or controls. No correlation was noted between the interval from the treatment and the MF, rather 9 patients with high MF ($>10\times10^{-6}$) had not received any treatment for more than 24 months. Fifteen out of 45 ALL but none of AML patients had high MF ($>10\times10^{-6}$) which persisted in those patients at the second determination. Because the elevation in MF persisted in patients for more than 24 months after the cessation of treatment, the authors speculated that anti-cancer treatment might have had mutagenic effects on lymphoid stem cells. Although the authors offer several suggestions on why only ALL, but not AML patients, had elevated MF one observation was that the ALL, but not AML patients, were given cyclophosphamide which has been shown to be mutagenic in other chemotherapy studies. Because the majority of patients also received radiotherapy it is difficult to make conclusions concerning the effects of chemotherapy only. Although the clonality of mutants was investigated, the number of subjects (5) and the number of mutant clones (3-9) analyzed was too small to make a definitive conclusion. The nature of mutations in 52 mutants from ALL patients was also investigated in this study and it was found that 23 % of mutants had structural alterations detected by Southern blot analysis compared to no such alterations in 38 mutants from controls. This indicated that induced mutations were mostly gross structural alterations.

Branda *et al.* (1991) analyzed 49 women with breast cancer treated with surgery alone or additional tamoxifen, radiotherapy, or multiple drug chemotherapy, which was

completed within 6 months after diagnosis for all but one patient. Samples were taken and analyzed at 6-months intervals for 2 years. The mean MF before treatment was $12.0\pm1.7x10^{-6}$ (CE=0.37±0.23) which was not significantly different from MF in healthy women. Overall, the mean MF increased significantly after the initial 6 months to $16.7\pm1.7x10^{-6}$ but then dropped gradually to the pre-treatment level during the next 12 to 24 months, although CE did not change significantly. When partitioned into treatment groups it was found that the MF was increased significantly ($18.2\pm1.7x10^{-6}$ compared to $11.1\pm1.4x10^{-6}$) after 6 months only in a group of 15 patients (age 46.9±9.3) who received combination chemotherapy with or without radiation or tamoxifen, but not in patients treated with surgery alone or patients treated with surgery, tamoxifen plus radiotherapy. This significant difference was due to 5 patients (33%) which had MF of more than 3 standard deviations from the mean of the initial level. However, only two of 16 patients treated with radiotherapy had MFs more than 3 standard deviations above the mean of the initial MF. It is worth noting, however, that the standard deviation of the mean initial MF was more than twice as large for radiotherapy than for chemotherapy patients. The increase in MF after chemotherapy was inversely related to the serum folate levels in the patients and the number of years of smoking. The CE did not change with treatment in any of the groups, although it was overall higher (above 50%) in patients who did not receive radiotherapy or chemotherapy and the mean MFs in these patients were correspondingly lower. Interestingly, the mean MF after 6 months was the same in patients treated with radiation and chemotherapy ($18.2\pm1.7x10^{-6}$), however because the initial pre-treatment mean MF in radiotherapy patients was higher ($13.2\pm1.9x10^{-6}$) than in

chemotherapy patients ($11.1\pm1.4x10^{-6}$), which was probably due to the fact that radiotherapy patients were older, the increase in MF in radiotherapy patients was insignificant. No investigation on clonality of mutants was done in this study.

In another study of breast cancer patients, the increase in the *hprt* MF could be wholly accounted for by the effect of radiation, while chemotherapy had no effect on the MF (Sala-Trepat *et al.*, 1990).

Ammenheuser *et al.* (1988) used autoradiographic *hprt* assay to study prospectively 5 multiple sclerosis patients (mean age 37, range 28-50) treated with cyclophosphamide infusions given for 2 h monthly for 6 months. The pre-treatment variant frequency *Vf* for 8 patients was $4.07x10^{-6}$, which was significantly higher than in 8 age-matched non-smoking healthy control subjects. This difference was attributed to smoking as 4 patients were smokers and their *Vf* was significantly higher than in non-smoking patients. The results showed that the *Vf* was significantly increased (8- to 20-fold) in patients 2 weeks after the first infusion but had fallen to pre-treatment level 4 weeks after the first infusion. Subsequent samples were taken after the treatment cycle and the rest period of approximately 4 weeks or just before the next infusion and the *Vf*, although still significantly elevated in some patients, were found to be less elevated than the *Vf* after the first treatment cycle. However, after 7-13 weeks after the completion of chemotherapy the *Vf* returned to pre-treatment level in all patients. The authors suggested that the transient nature of mutagenic response after cyclophosphamide treatment indicated rapid *in vivo* selection against the drug-induced *hprt* mutant cells as well as a balance between the introduction of new mutations and the toxic effects of the drug. The possibility that

the treatment could cause a high proportion of lethal multilocus deletions and that the mutant cells could have been diluted after the repopulation of blood with new cells was noted. Interestingly, no significant elevations in chromosome aberrations were detected at the post-treatment samples in these patients even after a total of 6,000-7,000 mg of cyclophosphamide given intravenously for 5-6 months (Ammenheuser *et al.*, 1991). By using the *hprt* clonal assay, Palmer *et al.* (1988) showed that cyclophosphamide was associated with the increased *hprt* MF in lymphocytes of patients with connective tissue disease, which was related to the duration of therapy.

Caggana *et al.* (1991) studied 3 cohorts of Hodgkin's disease patients (N=86, age 18-84). These included 18 previously untreated patients (age 29.6±10.3), 45 patients treated with radiation (2 to 183 months after the treatment) (age 35.1±12.8) and 25 patients including those treated with combined MOPP (mechlorethamine, vincristine, procarbazine and prednisone) chemotherapy and radiotherapy 9-174 months prior to sampling (N=23, age 34.3±14.1) and 2 patients who received only MOPP and ABVD (adriamycin, bleomycin, vinblastine and dimethyltriazenoimidazolecarboxamide) chemotherapy. The untreated patients had a significantly lower CE than controls (11.3% *vs.* 21.1%) and a higher, although not significantly, MF ($7.6x10^{-6}$ *vs.* $4.6x10^{-6}$). The radiotherapy treated patients also had a significantly lower CE (10.1±7.6) and a significantly higher MF ($15.6x10^{-6}$). For the third group of patients treated with radiotherapy and chemotherapy, the CE was again significantly lower than that for the controls (10.7±13.6%) and the MF was significantly elevated ($15.7±12.4x10^{-6}$). Interpatient variability was found in this study, where 17% of untreated patients had MF

above the upper 95% confidence limit ($12.6x10^{-6}$) for MF in controls, but 31% of radiation treated patients and 44% of radiation plus chemotherapy treated patients had MF greater than $12.6x10^{-6}$. This was correlated with the relative risk of secondary leukemia in a percentage of treated Hodgkin's disease patients which is highest in patients who received the most intense treatment. Interestingly, contrary to other studies, age, smoking, stage of diagnosis and gender did not appear to have effect on MF in any of the three groups of patients in this study, although subjects with a wide range of ages (18-80) were analyzed in the control. Gender and smoking were found to be significantly associated with MF only in control subjects. However, in non-smoker controls the gender was found to have no effect on MF. Also, contrary to other studies, there was no significant correlation between the time after the completion of treatment and MF. No investigation of clonality was done in this study.

Dempsey *et al.* (1985) studied 28 untreated patients with solid tumors (age 59±3), 14 untreated patients with lymphoma (age 52±4), and 27 patients with solid tumors (age 45±6) or lymphoma (age 55±3) treated with chemotherapy and/or radiotherapy. The mean MF for untreated patients ($6.7x10^{-6}$) was not different from the MF in the control subjects ($6.9x10^{-6}$ for solid tumor controls, N=23, age 50±3 and $5.3x10^{-6}$ for lymphoma controls, N=22, age 43±3). A significant increase in MF was detected in patients following chemotherapy ($19.57x10^{-6}$) and following chemotherapy and radiotherapy ($34.40x10^{-6}$). There was no significant difference between the effect of chemotherapy alone or chemotherapy plus radiation on MF. However, a great difference in CE was detected in various groups although no comment is made in the paper on its significance.

Thus, the CE in lymphoma controls, untreated and treated patients was 33.3, 14.6 and down to 6.5 respectively, while in solid tumor controls, untreated and treated patients the CE was 26.5 to 20.9 and 5.1 respectively. Therefore, the apparent increase in MF might have been confounded by changes in CE. Patients were treated with various multiple drug schedules at various intervals prior to the sampling, however, no investigation of the effects of individual drugs or the interpatient variability on MF has been done.

From the studies described above it can be concluded that the cancer chemotherapy can be associated with the induction of somatic mutation *in vivo* as measured by the *hprt* T-cell clonal assay. However, it is clear that even in the same group of patients treated with the same treatment regimen there is always an inter-individual variation so that in many patients the potentially mutagenic treatment does not necessarily associate with an increase in mutation. Because there are many confounding factors that may influence the measured *hprt* MF in an individual blood sample, it is unclear what determines whether the effect will be positive or negative. It appears that some chemotherapy regimens might be more mutagenic than the others and that the time of the measurement relative to the previous treatment might be important. Of all the confounding factors influencing the end result, perhaps one of the most important is the complex nature of T-cells (see below). Other factors such as apoptosis and individual susceptibility (drug metabolism, DNA repair) also might play a major role in the mutagenic response to chemotherapy.

7. T-cells as part of the immune system.

7.1. Development of T-cells.

During embryonic life, pluripotent stem cells migrate to the primary haemopoietic sites, the bone marrow, spleen and liver. Here they give rise to the precursors (committed

progenitor cells) of all blood cells, including lymphoid, myeloid and erythroid cell lineages. Pro-T-cells migrate to the thymus during foetal life. In the thymus T-cells rearrange their germ-line T-cell receptor (TCR) genes coding for the αβ and γδ molecules of the receptor heterodimer through the process involving recombination and somatic mutation. This result in the generation of a very large number of different T-cell clones each bearing a unique rearranged T-cell receptor. The cells that emerge from the thymus are known as naïve T-cells and they form the "virgin" lymphocyte pool. They are not fully differentiated, and retain the capacity for self-renewal. Thus, although the thymus is maximally active in neonatal life and involutes with age, the individual maintains a population of post-thymic T-cell precursors in the periphery for life. These cells reside mainly in the secondary lymphoid tissue (lymph nodes or spleen), where they remain for considerable periods, although they recirculate via the lymphatics to the blood at intervals. Following stimulation by an antigen, T-cells with a receptor specific to this antigen proliferate and differentiate as clones into either short-lived effector cells (such as cytotoxic or helper T cells) or long-lived memory cells constituting the memory cell pool. This cellular proliferation is the primary response. The resting memory cells circulate through the body allowing surveillance for invading microorganisms and upon exposure to the same antigen can recognize and attack it, but now in greater numbers. The activated cells again proliferate, and some differentiate into effector cells while other cells remain as memory cells, the so called "secondary response". The peripheral blood represents only about 1-2% of the total lymphocyte pool in normal adult humans. Lymphocytes continually enter and exit the blood stream from secondary lymphoid

organs, in addition to homing to other organs such as lungs or skin where they reside for varying periods of time.

Thus, it can be seen that diversity exists in the T-cell population circulating in the blood where the *hprt* MF is measured. In addition to undifferentiated post-thymic virgin cells and memory cells, both of which are long-lived (these constitute 90% of the T-cell population with a half-life of about 3 years, including some that have a lifespan of several decades) and retain capacity for further clonal expansion, many clones of fully differentiated, shorter-lived (remaining 10% of cells with a half-life of 1-10 days) effector T-cells of different types are also present (Cole *et al.*, 1994, Tates *et al.*, 1994).

7.2 T-cell subpopulations.

T-cells constitute about 70% of the blood lymphocyte population in normal donors (T lymphocyte pool is estimated to be $1\text{-}5x10^{11}$ cells for a normal healthy adult) (reviewed in Cole *et al.*, 1994). T-cells circulating in the blood are normally in the G_0 phase of the cell cycle (90% of cells). The two main subpopulations of T-cells are those expressing the CD4 molecule, which recognize antigen presented in association with major histocompatibility (MHC) class II molecules and which act predominantly as helper cells, and CD8-expressing cells, which recognize antigen in the context of MHC class I molecules and which are mainly concerned with the cytotoxic destruction of virally infected cells. Either class may act as suppressor cells, depending on the context in which antigen is presented. Both CD4+ and CD8+ cells have subsets. For example, virgin T-cells are CD45R+, while activated or memory cells are CD29+. Circulating T-cells also express other characteristic surface markers. For example, T-cells in G_0 phase express CD2 and CD5, while activated T-cells may be induced to express CD25, which forms

part of the high affinity interleukin-2 (IL-2) receptor which is important for clonal expansion. All mature immunocompetent T-cells express CD3, a complex of polypeptides involved in signaling cellular activation. Associated with CD3 is the antigen receptor molecule. This diversity of cells expressing different surface markers is partly generated by T-cell differentiation in the thymus during neonatal development and following antigen-dependent differentiation during the life of an individual.

7.3 Rearrangement of TCR genes.

The T cell antigen binding receptor (TCR) consists of heterodimer of two subunits. Each subunit in heterodimer is formed through the rearrangement of corresponding TCR genes. The specificity of a T cell is determined by the unique rearrangements of the TCR genes. In human peripheral blood most of the CD3+ cells express the αβ T cell receptor (Alexandre *et al.*, 1992). A smaller fraction of CD3+ T cells express the γδ T cell receptor (from 1 to 10% depending on individual with an average of 3-5%). Interestingly, although the αβ T cells never express the γ chain at the cell surface, most of them (about 98%) rearrange the γ locus on both alleles (Alexandre *et al.*, 1992). The gene segments are rearranged by the action of VDJ recombinase. One variable gene segment is joined to one of the joining gene segments attached to a constant gene. The VDJ recombinase enzyme has endonuclease, exonuclease and deoxynucleotidyl transferase activities. The enzyme recognises specific heptamer/nonamer sequences at the variable and joining gene segments and recombines them. Exonuclease and dioxynucleotidyl activities are responsible for the random sequence at the junction between variable and joining segments. The N region

consists of randomly added or deleted nucleotides, making the number of possible outcomes of the rearrangements many times larger.

7.4 Detection of T-cell clonality by the analysis of TCRγ-gene rearrangement.

Once the TCR genes are rearranged and the specificity of the T-cell is determined, all subsequent daughter cells will have the same TCR genes rearrangement and specificity. Any T cell with a particular set of rearranged TCR genes that responds to an activating signal will proliferate and form a clonally expanded population. If original cell sustains a mutation (let say at the *hprt* gene), the entire clonal population will have this mutation. In the mutation analysis, such as that performed by the *hprt* T-cell clonal assay, this would appear as an increase in MF and a "hot spot" in the mutational spectrum. It is, therefore, important to determine the clonality of the *hprt* mutants, especially, if an unusually high MF or a large proportion of identical mutations is detected in a blood sample. The analysis of the TCR genes rearrangements provides the means for determining the mutant clonality since, as described above, clonal mutants should have identical rearrangements of their TCR genes.

Methods that have utilized T-cell receptor gene rearrangement pattern for the analysis of clonal relationships depended on the β-, γ-, and δ-genes (reviewed in de Boer *et al.*, 1993). Both, Southern blotting of genomic DNA digests with probes specific for a T-cell receptor gene and the use of PCR amplification of a part of the rearranged TCR gene spanning the V-N-J junction in combination with analysis of the products on a denaturing gradient gel or by single-stranded conformation polymorphism electrophoresis (SSCP), have been used. Amplification of rearranged genes by PCR is primarily limited to the γ-

gene, since α- and β-genes are highly polymorphic, with possibly several hundreds of variable gene segments, while γ-gene has 14 variable genes only 6 of which (five belonging to one subgroup and are highly homologous) are expressed and two of each joining and constant gene segments. The simpler gene structure greatly reduces the number of possible combinations, however, diversity is maintained through the N region. This features permit the use of TCR γ-gene in PCR-based methods for the determination of mutant clonality. The consensus primers for the PCR amplification of the V-N-J junction can be easily designed and, at the same time, different clones can be distinguish due to their N-region diversity. De Boer *et al.* (1993) developed a modification of a PCR method where the analysis of restriction length polymorphism (RFLP) of fragments after enzymatic digestion of the PCR product containing the amplified part of the T-cell receptor γ locus is introduced to replace the denaturing gradient or SSCP gels.

7.5 Restriction fragment length polymorphism of the PCR-amplified TCR γ gene.

The human TCR γ-locus consists of variable V, joining J and constant C gene segments located on chromosome 7p14-p15 and spanning 160 kb (Figure 1.9) (Lefranc *et al.*, 1989, Bensmana *et al.*, 1991). There are 14 variable gene segments making up 4 subgroups. The number of VγI genes belonging to the first subgroup may vary from 7 to 10 per haploid genome, the 9-gene haplotype being the most frequent. Out of 14 variable genes there are 8 pseudogenes and only 6 genes are expressed. There are 5 joining and two constant gene segments. Five of the 6 active variable genes belong to one subgroup (VγI subgroup) and are highly homologous while the last active gene, Vγ9, belongs to another subgroup. Studies have shown that γδ T cells express preferentially Vγ9 gene (Sturm *et al.*, 1989). In

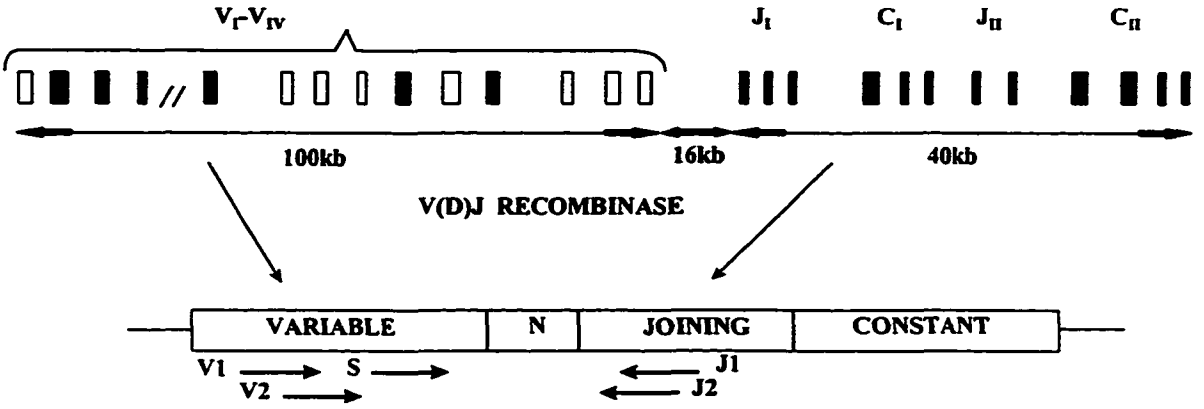


Figure 1.9 Schematic representation of TCR-γ gene genomic sequence with the locations of variable (V), joining (J) and constant (C) gene segments (empty and black rectangles) shown together with rearranged gene and approximate location of PCR and sequencing (S) primers used to amplify and sequence the TCR-γ gene rearrangements in the *hprt* mutants. Empty rectangles represent pseudogenes. (Adapted from de Boer *et al.*, 1993).

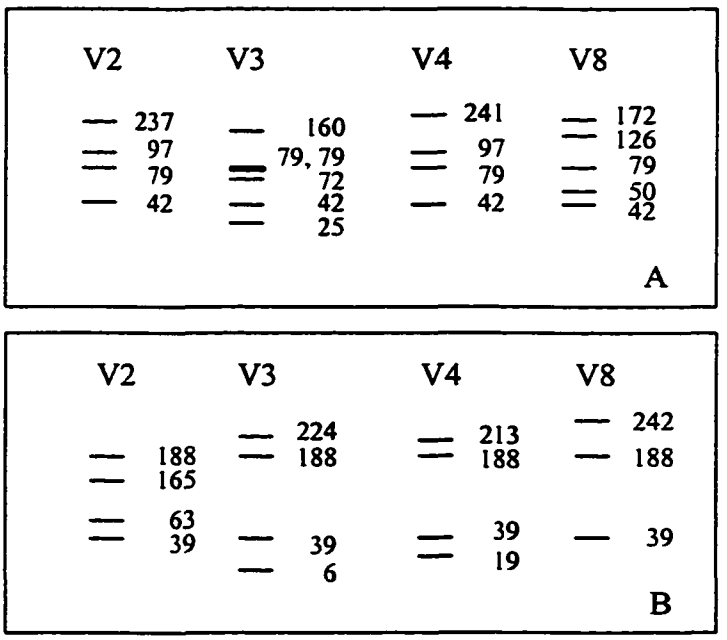


Figure 1.10 Schematic representation of the restriction fragments (lengths in bp) of the PCR amplified TCR-γ variable (V) genes with BstN1 (A) and RsaI (B). (Adapted from de Boer *et al.*, 1993.)

early ontogeny when gamma locus is active (8-13 weeks), most rearrangements involve TCR γ CI region (JP1, JP, J1 segments) (Zhang *et al.*, 1994). However, in most *in vivo*-sensitised alloreactive T-cell clones, rearrangements involved J2 segment with preferential use of V2 and V4 genes (Moisan *et al.*, 1989). The TCR-γ genes are rearranged in both αβ and γδ T-cells and some variable genes (VγI subgroup) were shown to be preferentially used in the rearrangements (Bourguin *et al.*, 1990).

Two sets of nested primers were designed that could amplify a fragment including most of the variable and joining segments (Bourguin *et al.*, 1990) (Figure 1.9). The two Jγ primers anneal to the Jγ1 and Jγ2 segments and can be used to amplify all TCR γ-gene rearrangements. The two Vγ primers anneal to sequences of the Vγ1-8 segments belonging to the Vγ subgroup I and can be used to amplify the great majority of TCR γ-gene rearrangements (de Boer et al., 1993, Bourguin *et al.*, 1990). The outer pair of primers generates a product of about 500 bp while the inner pair generates a product of about 460 bp.

The polymorphisms present in different variable genes are demonstrated in the presence and absence of a number of indicative restriction enzyme sites. After digestion of the PCR product with two restriction enzymes (BstN1 and RsaI), each variable gene gives a particular set of different length fragments (Figure 1.10). The fragment of 42 bp in the BstN1 digestion spans the junction between the variable and joining segments and the number of nucleotides that are deleted or added by the VDJ recombinase determines its length. This junction is also included in the 188 bp fragment after the RsaI digestion. After resolving the digestion fragments on the low melting point agarose gel, the pattern of the

fragments can be seen. The appearance of two distinct patterns after digestion of the PCR amplified TCR γ-gene from two mutants with two enzymes is taken as an indication that the mutants are not clonally related. In contrast, if the patterns are the same, the mutants are assumed to be clonal. Perhaps, the most informative of all fragments is the small 42 bp fragment obtained after digestion with BstN1. The small size of the fragment makes it possible to detect the addition or deletion of small numbers of base pairs at the N region even if mutants used the same variable genes for their TCR γ-gene rearrangement.

8. *hprt* gene as a mutagenesis target.

8.1 Catalytic activity, gene structure and mRNA levels.

Hypoxanthine phosphoribosyltransferase (HPRT) catalyzes the reaction of 5'-phosphoribosyl-1-pyrophosphate (PRPP) and the purine bases hypoxanthine and guanine in the formation of 5'-IMP and 5'-GMP respectively. Together with adenine phosphoribosyltransferase (APRT), which catalyzes a similar salvage reaction with adenine, these enzymes are providing cells with an alternative "salvage" pathway of purine nucleotide biosynthesis. Ninety percent of free purines in humans are recycled and "salvage" pathway plays a crucial role in maintaining the intracellular purine nucleotide pools (Stout *et al.*, 1985). In addition to hypoxanthine and guanine, HPRT can bind and ribosylate a wide range of toxic purine analogues such as 6-TG, a characteristic exploited in mutagenesis studies for the isolation of HPRT$^-$ cells. The isolation of mutants is further facilitated by the fact that the *hprt* gene is located on mammalian X-chromosome and is hemizygous in males or functionally hemizygous in females due to X-chromosome inactivation early in the female development.

HPRT is found as a soluble, cytoplasmic enzyme and accounts for 0.005-0.04% of total protein. It is composed of four identical subunits of molecular weight 24,500. HPRT has been detected in all somatic tissues at low levels (0.005-0.01% of total mRNA). Significantly higher levels of HPRT are found in the central nervous system (0.02-0.04% of total mRNA) with basal ganglia of the brain having the highest level of HPRT activity and the correspondingly lowest *de novo* synthesis of purines. HPRT action requires magnesium and the specific mechanism of catalysis is greatly influenced by the Mg^{2+}/PRPP ratio.

HPRT deficiency in humans is associated with two clinically recognizable disorders: a complete deficiency of HPRT results in the Lesch-Nyhan syndrome, severe neurological disorder; a partial deficiency results in hyperuricemia and a form of gouty arthritis (Stout *et al.*, 1988).

Human *hprt* gene is approximately 42 kbp in length and consists of 9 exons making up a 654 bp cDNA. A complete sequence of 57 kbp of the human *hprt* gene has been determined (Edwards *et al.*, 1990). *hprt* gene belongs to a family of housekeeping genes. It lacks the conventional 5' transcriptional regulatory sequences such as TATA and CAAT boxes and has extremely GC-rich sequences and several hexanucleotide motifs at the 5' end of the gene (Kim *et al.*, 1986).

In resting G_0 lymphocytes the *hprt*-RNA level is very low (approximately one mRNA molecule per cell). In phytohemagglutinin (PHA) stimulated lymphocytes the *hprt*-RNA levels increase 10-20-fold, while the enzyme activity increases 5-fold. It was concluded that the enhanced expression of *hprt*-RNA in proliferating lymphocytes depends on continuous growth stimulation and seems to be associated with a high transcriptional

activity and turnover of the RNA. In contrast, the enzyme activity is relatively stable, and less sensitive to alterations in growth conditions (Steen *et al.*, 1991).

8.2 *hprt* mRNA levels and enzyme activity in 6TG resistant mutants.

Steen *et al.* (1993) studied HPRT activity and RNA phenotypes in 6TG resistant human T-cells. They found that *hprt* mutation often leads to decreased steady state levels of *hprt* mRNA in 6TG resistant T-cell clones. Many different types of *hprt* mutation can have this effect. Reduced mRNA levels were found in all three nonsense mutations, 4 out of 5 splicing mutations, both of two clones with genomic alterations, 3 out of 5 missense mutations and 1 out of 4 frameshifts caused by 1-4 bp deletions (Steen *et al.*, 1993). The authors proposed that the *hprt* mutation can affect the transcription, transport to or the stability of the *hprt* mRNA in the cytoplasm. In another study with CHO cells, 80% of nonsense mutants, but only 14% of missense mutants had <50% of the *hprt* mRNA concentration found on parental CHO cells (Manjanatha *et al.*, 1994). Further, nonsense mutations in the internal exons of the gene were strongly associated with a significant reduction in mRNA levels (<16% of parental on average) as opposed to the nonsense mutations in the extreme 5' and 3' regions of the gene. Also, the proportion of mutants producing multiple cDNA PCR products was much greater for mutants having nonsense mutations (especially mutations in exons 3,4 and 5) than for mutants with missense mutations (Manjanatha *et al.*, 1994). The mean enzyme activity in 16 6TG selected clones was less than 1% of the mean in unselected clones, however intermediate and high enzyme activity and normal *hprt* mRNA levels were found in 2 6TG selected clones where no *hprt* mutation was detected (Steen *et al.*, 1993). The significant HPRT enzyme activity was found in the higher proportion of mutants (20%-50%) when less stringent

selection conditions were used in another study (1 μg/ml *vs.* 2 μg/ml). Thus, some cell clones with residual HPRT activity may escape 6TG selection. It was proposed that some mutants can lose their catalytic activity towards 6TG but not towards hypoxanthine because of impermeability to 6TG due to faulty transportation across the cell membrane (Steen *et al.*, 1993). Several clones with very low *hprt* mRNA levels were found to yield *hprt* cDNA by PCR amplification (Steen *et al.*, 1993).

8.3 *hprt* mutational spectra in humans *in vivo*.

hprt gene is a good mutagenesis target because it is sensitive to different types of mutation and it has many mutable sites so that a large variety of mutagenic DNA lesions can be detected by the *hprt* T-cell clonal assay. The spectrum of the *hprt* mutations in healthy adult population consist of base substitutions (around 50% of mutations), deletion/insertion type mutations (around 40%) including +/- 1 frameshifts, small (2-200 bp), large deletions, and duplications, and complex mutations (2%) involving concomitant deletions and insertions of several base pairs (Burkhart-Schultz *et al.*, 1996). It appears that many bases of the *hprt* cDNA coding region (657 bp) are mutable (291 different base substitutions were observed at 208 bases) providing that the *hprt* target is far from being saturated since many novel positions affected by base substitutions are being recovered (Cole *et al.*, 1994, Curry *et al.*, 1996). The *hprt* target contains 411 positions where single base pair substitutions lead to amino acid change and mutations in those positions should be recoverable. A vast majority of base substitutions lead to missense mutations (88%), while a small proportion of base substitutions (0.3%) does not lead to any amino acid change (Cole *et al.*, 1994). It was proposed that the latter class of

mutation may express phenotypically due to the preferential codon usage or due to decreased stability of the resulting *hprt* mRNA (Cole *et al.*, 1994).

Several "hot spots" for base substitutions (positions 197, 508, 617) were identified. Spontaneous deamination of methyl-CpG may be implicated in the creation of a "hot spot" at base pair 508 since all mutations are C>T transition resulting in nonsense mutation. There is a bias towards base substitutions at the GC base pairs and GC>AT transition is by far the most common (around 34% of base substitutions) while other substitution types are relatively evenly distributed (9-16% of all substitutions) (Curry *et al.*, (submitted)).

Most of the frameshifts occur at or adjacent to sequences containing two or more consecutive bases and most of the deletions are flanked by short direct repeats suggesting a slipped mispairing model in their formation. Seven percent of *hprt* mutations in adult males are total deletions of the *hprt* gene (Fuscoe *et al.*, 1992 (2)). The maximum recoverable deletion size is at least 3.5 Mb (1.4 Mb 3' deletions telomeric and at least 2.1Mb 5' deletions centromeric to the *hprt* gene) indicating that large deletions at the hemizygous X-linked *hprt* gene are tolerated (Lippert *et al.*, 1994).

Mutations affecting exon splicing are found in around 40% of all *in vivo* mutations and are caused by base substitutions or small deletions either in exon sequences or splice sites as well as by genomic deletions of regions covering the whole exon. Mutations resulting in the loss of exons 4 and 8 appear to occur at the highest frequency suggesting that important catalytic sites are located at sequences that regulate splicing of these exons. Alternatively, these sequences are either larger than those of other exons, or these sequences are especially prone to mutation (Cole *et al.*, 1994).

In adults, only 15% of *hprt* mutations have structural alterations detectable by the Southern blots, while in the fetus 66%-85% show alterations of which 40%-75% are deletions of exons 2 and 3. Although this class of mutation represents only 2% of the total mutations in adults compared to 40% or more in newborns, the frequency of this mutation is similar in newborns and adults. The reason why newborns have a higher percentage of exon 2 and 3 deletions is that this mutation becomes "diluted" with other induced accumulated mutations in adults who have 10-fold higher overall *hprt* MF (Cole *et al.*, 1994). Illegitimate recombination mediated by VDJ recombinase during T-cell development was implicated in the exon 2 and 3 deletion mutations because VDJ-recombinase heptamer and nonamer recognition sequences were identified at the deletion breakpoints (Fuscoe *et al.*, 1992 (1)).

Over two thousand human *hprt* mutants from both *in vitro* and *in vivo* sources have been sequenced to date by several laboratories. A database containing all known human *hprt* mutations is being maintained (MutaBase Software, Durham, NC). The mutations characterized *in vivo* include somatic mutations derived from normal non-exposed individuals, smokers, patients receiving chemotherapy and radiotherapy, patients with DNA repair disorders and germinal *hprt* mutations in Lesch-Nyhan patients. In addition, *hprt* mutations generated *in vitro* by agents including ENU, formaldehyde, ICR-191, MNNG, cyanoethylene oxide, cisplatin, BPDE, UV light, nucleotide pool imbalance and X-rays have been sequenced (Cole *et al.*, 1994). While *in vitro* exposures can produce distinct *hprt* mutational spectra characteristic of DNA damage or adducts specific to the genotoxic agent, *in vivo* induced spectra do not necessarily reflect a mutational signature of a particular mutagen. Smoking was shown to have no effect on the *hprt* mutational

spectra (Curry *et al.*, (submitted)). A few studies have determined mutational spectra in other exposed populations such as cancer patients after radio- and/or chemotherapy and people accidentally exposed to ionizing radiation (Skandalis *et al.*, 1995, Skandalis *et al.*, 1997, Hirota, *et al.*, 1993). It appears that occupational and environmental exposures are unlikely to produce a mutational signature on the *hprt* spectra. The reasons for this are ambient levels of exposures that produce only marginal increase in MF and the complexity of the spontaneous *in vivo hprt* spectra with a wide variety of mutation types and a high number of mutable sites.

9. Introduction to the project.

Cancer chemotherapy has greatly improved the survival prospects of patients. Two major drawbacks of this treatment, however, are toxicity and risk of secondary malignancy. Most currently in use chemotherapy drugs exert their effect on cell multiplication and tumor growth by interfering with synthesis or function of macromolecules such as DNA, RNA and proteins. While cancer cells are more sensitive to the cytotoxic effects of these drugs, normal cells in the body, especially those in active, rapidly dividing tissues such as bone marrow and mucous membrane cells, are also susceptible to the deleterious effects of toxic chemicals. The dangerous complication resulting from these effects in normal cells is the development of secondary treatment-related malignancy several years after the initial chemotherapy for the primary cancer.

It is believed that secondary malignancy arise as a result of genotoxic effects of chemotherapy agents, primarily DNA mutation. Most chemotherapy agents are known mutagens. During chemotherapy mutations may be induced in proto-oncogenes and tumor suppressor genes of the normal precursor cells in the body and these may be the

initial events leading to the development of secondary cancers later on. The understanding of this process is important not only in the possible prevention of secondary cancers by, for example, using less carcinogenic drugs and drug regimens. It is also important in the understanding of mechanisms by which primary malignant diseases occur since endogenous and environmental carcinogens implicated in the pathogenesis of human cancer and chemotherapy agents have similar modes of action.

The purpose of this research is to investigate the genotoxic consequences of chemotherapy in cancer patients. The primary focus is on DNA mutation. Two groups of patients were chosen: SCLC patients treated with topoisomerase II inhibitor etoposide and MM patients treated with alkylating agent melphalan and prednisone. Most chemotherapy treatments include multiple drug regimens that can complicate the results on genotoxicity of chemotherapy treatment. Because patients here received only one cytotoxic drug, it was possible to determine the effect of this particular agent. Etoposide and melphalan are among the most leukemogenic currently used chemotherapy agents. The *hprt* T-cell clonal assay was used to determine the *hprt* MF and mutational spectra in each individual patient before and several times during the treatment cycles. Results of the assay before and after treatment were then compared so that the effect of the individual drug could be elucidated.

Specific chromosomal abnormalities of the MLL gene were also investigated in a group of SCLC patients. Rearrangements of the MLL gene are the predominant rearrangements found in sAML after chemotherapy with topoisomerase II inhibitors and in some *de novo* childhood leukemias. Because sequences within the bcr of the MLL gene are believed to be hypersensitive to the damage by topoisomerase II inhibitors, it is

important to determine whether etoposide can directly induce MLL gene rearrangements found in sAML. Using fluorescent *in situ* hybridization (FISH) with a probe spanning the bcr of the MLL gene, the blood cells of SCLC patients were screened for the appearance of MLL gene rearrangements after etoposide chemotherapy.

CHAPTER II. MUTATION FREQUENCY AND SPECTRUM IN LYMPHOCYTES OF SMALL CELL LUNG CANCER PATIENTS RECEIVING ETOPOSIDE CHEMOTHERAPY

1. Abstract.

Etoposide, a topoisomerase II inhibitor, is a chemotherapeutic agent used in the treatment of a wide variety of neoplasms, including small cell lung cancer, germ cell cancer, testicular cancer, acute leukemia, and lymphoma. Although it has proven valuable, etoposide is also a known mutagen, and has been implicated as a causative agent of treatment-related secondary acute myeloid leukemia (sAML). We have investigated the induction of mutation following etoposide treatment *in vivo* using the hypoxanthine-guanine phosphorybosil transferase (*hprt)* T-cell cloning assay in small cell lung cancer (SCLC) patients receiving single drug etoposide chemotherapy. This Chapter presents results on monitoring 12 patients (mean age 74.8 ± 6.0; range 66-83) before, during, and after chemotherapy. The treatment regimen included up to 6 cycles of oral etoposide given in 50 mg tablets twice daily for 10-14 days separated by two weeks of rest. Peripheral blood samples were collected on the first day of each cycle prior to treatment. Patients received 1-6 etoposide cycles and were followed for 0.7-5.3 months after the start of chemotherapy (total etoposide dose 1.4-8.4 grams). Results from the pooled data show no significant increase in the *hprt* mutant frequency (MF) (pre-treatment mean 46 ± 38 $\times 10^{-6}$ versus 55 ± 46 $\times 10^{-6}$ post-treatment), although considerable inter-patient variability was observed. 228 mutants out of total 424 selected were analyzed by sequencing *hprt* cDNA. Spectra of 56 pre-treatment and 147 post-treatment

mutations revealed a significant enhancement of AT > TA transversions and a concomitant decrease in the number of GC >TA transversions in post-treatment spectrum when compared with pre-treatment or control spectra. No evidence for the induction of gross deletions or rearrangements was found in the spectra of mutants recovered from patients after etoposide treatment. The lack of the enhanced MF after treatment suggests that the etoposide chemotherapy was not particularly effective in inducing mutation as measured by the *hprt* assay. It is proposed that mutated cells are eliminated through apoptosis due to accumulated DNA damage.

2. Introduction.

Cancer patients receiving chemotherapy treatment are exposed to high doses of cytotoxic and often genotoxic drugs. These patients are at risk for the development of a secondary cancer several years after a successful regimen, which is believed to result from treatment (Smith *et al.*, 1994). A particular type of monocytic leukemia sAML is a most common secondary cancer among long-term survivors who receive Topo II inhibitors such as etoposide with the result that up to 12% of patients receiving epipodophyllotoxins later develop sAML (Smith *et al.*, 1994).

Leukemic cells from more than 50% of the Topo II inhibitor-related sAML display cytogenetic abnormalities of chromosomal band 11q23 (Domer *et al.*, 1995). The same region at 11q23 is also involved in rearrangements in the majority of some classes of *de novo* childhood leukemia (Bernard *et al.*, 1995). Although translocations in leukemia involve different genes on 11q23, the majority of 11q23 translocations involve *MLL* gene (Rowley, 1993). The *MLL* (also called *ALL-1* or *HRX*) gene and its 8.3kb breakpoint cluster region on 11q23 involved in sAML and *de novo* leukemia have been

characterized (Rowley, 1993, Gu *et al.*, 1994). The significance of this region in the pathogenesis of leukemia is not understood. Several studies have suggested the involvement of Alu repeats (Gu *et al.*, 1994, Schichman *et al.*, 1994), the action of VDJ recombinase (Gu *et al.*, 1992, Felix *et al.*, 1995 (1,2)), and Topo II (Domer *et al.*, 1995, Felix *et al.*, 1995 (2), Negrini *et al.*, 1993) in the pathogenesis of 11q23 rearrangements. Topo II DNA recognition sequences have been identified at the breakpoints of the 11q23 rearrangements involved in leukemia (Negrini *et al.*, 1993) and Topo II-inhibitor induced cleavage sites *in vitro* have been shown to coincide with the sequences in the *MLL* gene involved in some rearrangements in *de novo* and secondary leukemia (Domer *et al.*, 1995, Felix *et al.*, 1995 (1)). Coincidence of Topo II cleavage sites and breakpoints in leukemia rearrangements suggests the involvement of Topo II inhibitors in the leukemogenic process in sAML.

Etoposide is a non-intercalating Topo II inhibitor which acts by blocking the re-sealing function of Topo II resulting in single and double strand breaks, inhibition of DNA synthesis and arrest of cells in the late S or early G_2 phase of the cell cycle (Liu 1989, Anderson *et al.*, 1994, Ferguson *et al.*, 1994). The mode of etoposide cytotoxicity is not completely understood, however, evidence suggests that the initial event is the DNA strand breakage mediated by Topo II. This is followed by secondary DNA fragmentation and apoptosis (Anderson *et al.*, 1994, Ferguson *et al.*, 1994). The genotoxic and clastogenic effects of etoposide *in vitro* are well documented. Etoposide mutagenesis has been studied in diverse systems (Anderson *et al.*, 1994, Ferguson *et al.*, 1994). Etoposide primarily induces large DNA deletions and rearrangements. However, it remains unclear what kind of rearrangements and/or mutations are induced during

chemotherapy. There have been no reports on the etoposide mutational spectrum in human cells.

The *hprt* gene is a genetic marker in a well-developed T-cell cloning assay, which is the most frequently used in human monitoring studies of mutation. A number of laboratories have been involved in analyzing *hprt* mutation frequency and spectra in various systems *in vitro* and *in vivo* (reviewed in Cole *et al.*, 1994). As a result of these efforts, extensive data became available not only on the background *hprt* mutation frequency in an unexposed healthy human population, but also on the sensitivity of the assay to detect changes in mutation frequency in individuals with cancer-prone syndromes and DNA repair disorders as well as the effects of various potentially mutagenic exposures in humans including smoking, cancer therapy, environmental and workplace exposures. The human *hprt* mutant database currently contains published information on 2169 mutations detected in 1973 *hprt* spontaneous and induced mutants obtained in various cell systems from *in vitro* and *in vivo* studies by many laboratories (available from MutaBase software, Durham, NC).

Several studies on mutation induction during chemotherapy using the *hprt* T-cell clonal assay have been conducted (Branda *et al.*, 1991, Tates *et al.*, 1994, Hirota *et al.*, 1993, Ammenheuser *et al.*, 1988, Caggana *et al.*, 1991, Dempsey *et al.*, 1985). However, these are often complicated by the use of multiple treatments. In this study we report on a group of SCLC patients treated with only a single drug etoposide. This allowed us to investigate possible mutagenic consequences of etoposide treatment on mutation frequency as well as mutational spectrum using the *hprt* T-cell cloning assay.

3. Materials and Methods.

Patients and sample collection. Blood samples were obtained after informed consent from SCLC patients at the Vancouver Island Cancer Clinic. The schedule for sample collection was dictated by the treatment regimen. Although patients varied regarding the number, timing and duration of the treatment cycles, they generally received 4 cycles of 50 mg oral etoposide twice a day for 2 weeks separated by 2 weeks of rest. Samples were collected on the first day of each cycle before treatment, so that the post-treatment samples were collected at the end of the rest period or approximately 2 weeks after the completion of the previous cycle. The pre-treatment sample served as a control for the individual patient. A questionnaire with relevant information including age, smoking history, ethnic origin and occupation was completed for each patient. Information concerning 12 patients included in this study is presented in Table 2.1.

Table 2.1. Information on 12 SCLC patients undergoing etoposide chemotherapy.

Patient ID	Age (years)	Sex	Smoking (1pack/day× years)	N[a]	Blood sample time[b] (months)	Total dose[c] (g)
1E	80	M	45	2	0-1.7	0-2
5E	79	M	unknown	4	0-0.8-2.8-4.3	0-1.5-4.3-5.7
7E	67	M	30	4	0-1-2.4-4	0-1.4-3.1-4.1
21E	80	M	unknown	1	0-0.7	0-1.4
22E	66	M	8 in 1960s	4	0-3.3	0-5.6
8E	68	M	38	6	0-5.3	0-8.4
14E	80	F	55	4	0-1-4	0-1.4-5.6
15E	83	M	60	2	0-0.7-1.3	0-1.4-2.8
16E	75	F	50	3	0-2.2	0-4.2
17E	73	F	60	2	0-1.6	0-3.1
9E	69	M	45	1	0-1	0-1.4
24E	78	M	not since 1945	2	0-0.7-1.3	0-1.4-2.8
range	66-83			1-6	0.7-5.3	1.4-8.4
mean	75±6					

[a] N- number of etoposide treatment cycles.
[b] Time 0 is the first day of the first etoposide cycle.
[c] Total dose of etoposide received by the patients at the time of each sample collection.

Most patients were heavy smokers for much of their lives (30-60 years). The number of post-treatment samples varied among patients (Table 2.1). This reflected the individual patient's general condition and their availability for sample collection. Two patients (21E and 9E) were followed for only a single round of treatment, four patients (1E, 15E, 17E, and 24E) were followed for 2 cycles, one patient (16E) was followed for 3 cycles, four patients (5E, 7E, 22E, and 14E) were followed for 4 cycles and one patients (8E) was followed for 6 cycles (Table 2.1). The time for which the patients were followed ranged from 0.7 to 5.3 months after the start of chemotherapy and the patients received a total of 1.4 to 8.4 grams of etoposide during the follow up period (Table 2.1).

Approximately 30 ml of blood was collected from patients in 4 Leucoprep tubes (Becton Dickinson) and the MNC fraction was separated by centrifugation following the manufacture's protocol. MNCs were washed twice first in RPMI-1640 (HyClone), then in media containing 10% CBS (HyClone), RPMI-1640 supplemented with 4% of mixture containing 2493 U/ml penicillin, 1917.5 U/ml streptomycin (Sigma), 50 mM L-glutamine (Sigma) and 45 mM pyruvic acid (Sigma) and then counted using a hemocytometer. Samples were cryopreserved in 40% RPMI-1640, 50% CBS and 10% DMSO (Sigma) at a concentration $3\text{-}10\times10^{-6}$ cells/ml and stored in liquid nitrogen. In several instances (Table 2.2) and especially when the cell counts were below 5×10^{6} freshly isolated cells were pre-incubated with the mitogen, PHA (HA 17, Murex Diagnostics, England) immediately. When cryopreserved cells were used, the samples from an individual patient

Table 2.2 Results of the *hprt* T-cell cloning assay for 12 SCLC patients undergoing etoposide chemotherapy.

Sample no[b].	Non-selective Positive wells	Non-selective Total wells	Selective Positive wells	Selective Total wells	CE	MF x 10^{-6}	95% Confidence interval Upper ($x10^{-6}$)	95% Confidence interval Lower ($x10^{-6}$)	Fresh Cells Y/N[c]	No. of cells[a] $x10^{6}$
1 E-0	55	192	5	264	0.11	17	42	7	N	9
1 E-2	37	192	17	400	0.07	61[d]	108	34	N	12
5 E-0	21	192	7	136	0.04[e]	137[d]	322	58	N	7.5
5 E-1	10	192	20	848	0.02[e]	134[d]	286	63	N	31
5 E-3	17	192	43	888	0.03[e]	161[d]	282	92	N	13
5 E-4	56	192	75	960	0.11	71[d]	100	50	N	28.6
7 E-0	44	192	8	140	0.09	68[d]	144	32	N	8
7 E-1	32	192	3	140	0.06	36	116	11	N	7
7 E-3	21	192	20	536	0.04[e]	98[d]	182	53	N	15.6
7 E-4	36	192	5	160	0.07	46[d]	117	18	N	18
8 E-0	36	192	11	224	0.07	73[d]	143	37	N	15
8 E-6	65	192	17	264	0.14	48[d]	82	28	N	13.6
9 E-0	63	192	1	88	0.13	9	62	1	N	4
9 E-1	19	192	1	64	0.03[e]	45[d]	339	6	N	5.2
14 E-0	11	192	2	140	0.02[e]	73[d]	330	16	N	7
14 E-1	42	192	7	326	0.08	26	59	12	Y	6.5
14 E-4	30	192	3	344	0.06	15	51	5	N	11.6
15 E-0	54	192	8	480	0.11	15	32	7	N	40
15 E-1	53	192	6	340	0.11	17	38	7	N	20
15 E-2	30	192	12	1300	0.06	16	32	8	Y	28
16 E-0	71	192	23	864	0.15	18	28	11	N	40
16 E-3	104	192	17	688	0.26	10	16	6	Y	10.5
17 E-0	47	192	4	168	0.09	26	71	9	N	7.2
17 E-2	76	192	24	480	0.17	31	48	19	N	25
21 E-0	47	192	3	52	0.09	63[d]	204	20	Y	1.4
21 E-1	5	192	6	464	0.01[e]	148[d]	485	45	Y	20
22 E-0	76	192	13	248	0.17	32	58	18	Y	3.3
22 E-4	96	192	17	224	0.23	34	38	10	Y	4.4
24 E-0	92	192	20	360	0.22	26	43	16	N	20.6
24 E-1	117	192	13	184	0.31	23	42	13	N	11.7
24 E-2	75	192	13	248	0.17	33	59	18	N	11.6

[a] No. of cells recovered from the blood sample.

[b] E-0, pre-treatment sample; E-1 and E-6, samples obtained after the first and sixth etoposide cycles respectively.

[c] N, no; Y, yes

[d] MF of > $40x10^{-6}$; [e] CE of < 5%

were pre-incubated on the same day to minimize variability due to media and handling (samples 1E-0 and 1E-2 were cultured separately (Table 2.2)). In 7 cases samples were split and the experiments were performed either with fresh and cryopreserved cells or twice with cryopreserved cells, thus permitting the comparison of the results.

Cell culture. The procedure for the T-cell culture was based on the protocols described elsewhere (Curry *et al.*, 1993). Fresh or washed thawed cells were pre- incubated in 10 ml of supplemented RPMI-1640 medium containing 20 % HL-1 (Ventrix), 2.5% CBS, 2.5% human AB serum (Gibco/BRL), and 0.25 μg/ml of PHA for approx. 48 hours. Cells were then counted and plated in 96-well microtiter plates at a density of 3 cells/well for the determination of CE in non-selective medium containing supplemented RPMI-1640, 20% HL-1, 2.5 % CBS and 2.5% human AB serum, 20 U/ml of recombinant human IL-2 (Cellular Products Inc.), 0.15 μg/ml PHA and 1% fungizone (Gibco/ BRL). To select for mutants 10^{-5} M of 6-TG (Sigma) was added to the non-selective medium and 10^4 cells/well were plated in 96-well plates. 10^4 lethally irradiated feeder cells (RJK 853 lymphoblastoid cell line derived from a Lesch-Nyhan patient with a total genomic deletion of *hprt* gene) were added to each well in selective and non-selective plates. The plates were placed in a humidified atmosphere at 37°C and 5% CO_2 on a sloped (5°) shelf and were rotated 180° after 11 days incubation. After 14 days the plates were scored visually under an inverted phase contrast microscope for wells that contained expanded colonies. The numbers of positive and negative wells were determined for each plate and CE and MF were calculated according to formulas:

$$CE = -\ln(\text{proportion of negative wells})/n_0 \text{ and}$$

$$MF = -\ln(\text{proportion of negative wells})/n_1/CE,$$

where n_0 is the cell density plated in non-selective medium and n_1 is the cell density plated in selective medium. Mutant clones were transferred to 24-well plates in 1 ml selective growth medium and after 5 days approximately $6x10^4$ cells were removed, washed in Dulbecco's Phosphate-Buffered Saline (Gibco/BRL) and aliquoted into pellets of 2,000 cells for RT-PCR. The remaining cells from mutant clones were expanded, transferred to 4-well plates and pellets of 200,000 cells were prepared for multiplex PCR analysis. The remaining cells were expanded further to 1- 10 million cells and were cryopreserved in RPMI, 50% CBS and 10% DMSO and stored in liquid nitrogen for later use.

RT-PCR. The cell pellets of 2,000 cells were resuspended in 5µl of RT cocktail (21) containing 2 U of Mo-MLV reverse transcriptase (Gibco/BRL) and incubated at 37°C for 1 hour. Then, 45 µl of the first PCR reaction cocktail (Curry *et al.*, 1993) was added to each RT reaction. The first PCR reaction was performed according to the program: initial denaturation at 95°C for 5 minutes; 30 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 1 min; and the last extension at 72°C for 5 min. A second PCR reaction was performed in a total volume of 25 µl using 1µl of the first PCR product and the same PCR cocktail but with nested primers (Curry *et al.*, 1993). The same PCR program was used except that the annealing temperature was 55°C. 10 µl of this reaction was run on a 1% agarose gel stained with ethidium bromide. When cDNA was obtained, the second PCR reaction was repeated 3 times in a 100 µl reaction volume. DNA was purified from the 300 µl of the second PCR product using

Wizard PCR Preps (Promega) according to the manufacture's protocol and used for sequencing. The concentration of the template was measured using a TKO 100 Fluorometer (Hoefer Scientific Instruments, San Francisco) according to the manufacture's protocol.

Sequencing. The sequencing was performed on ALF automated DNA sequencer (Pharmacia) using ReadyMix Gel-ALF Grade (Pharmacia) and AutoCycle Sequencing kits (Pharmacia). The following PCR reaction was used: initial denaturation at 94°C for 2 min; 25 cycles of denaturation at 94°C for 20 sec, annealing at 50°C for 10 sec, and extension at 72°C for 1min. For sequencing cDNA, five primers, two forward (primer A: 5'-TGA GCA GTC AGC CCG CGC G-3'; primer B: 5'-ATG ACC AGT CAA CAG-3') and three reverse (primer C: 5'-ATC ACT ATT TCT ATT CAG TGC-3'; primer D: 5'-TGC GAC CTT GAC CAT CTT T-3'; E: 5'-AGG ACT CCA GAT GTT TCC -3') were used. The primers were selected to ensure the sufficient overlap of the sequences for the complete and accurate sequence determination. For sequencing genomic DNA, primers were used according to Gibbs *et al.* (1990) and synthesized by Dalton Chemicals (Toronto).

Multiplex PCR. When sequencing of the *hprt* cDNA showed missing exons or other splice errors, multiplex PCR (Gibbs *et al.*, 1990) was used in some cases to amplify genomic DNA to determine the underlying mutation.

Pellets of 200,000 cells were resuspended in 200 µl lysis buffer (Gibbs *et al.*, 1990) and incubated at 56°C for 90 min followed by 10 min in 96°C. 10 µl of crude cell extract (10,000 cells) was used per reaction. Usually in one reaction three *hprt* exons were

amplified simultaneously, one which was missing or altered in the cDNA, and the other two which were intact and were used as controls. PCR reactions were performed in a total volume of 50 μl with 3 pairs of primers (Gibbs *et al.*, 1990). Samples were overlaid with 20 μl mineral oil, placed in the thermocycler and heated to 94°C. After 6 min of hot start, 5U of Taq polymerase was added and PCR was performed for 35 cycles of 94°C - 30 sec, 61°C - 50 sec, 68°C - 2 min. The last extension time was 9 min. 20 μl of the PCR product was loaded on a 1% agarose gel and the exons were visualized.

Statistical Analysis. Multiple regression, χ^2 and t-test analysis were done using Statistica (Statsoft, Tusla). The spectra comparisons were done using χ^2 test assuming the expected equal proportion of mutants belonging to a specific mutation class in a total number of mutants in each spectrum being compared.

4. Results.

Mutant Frequency. A total of 31 pre- and post-treatment samples were analyzed (Table 2.2). The analysis of the pooled data indicates no significant effect of etoposide treatment on either CE (t=0.02, p=0.99) or on *hprt* lnMF (t=-0.57, p=0.6) (Table 2.3), although considerable inter-patient

Table 2.3 Comparison of the pre- and post-treatment *hprt* CE and MF for SCLC patients who received etoposide chemotherapy.

		pre-treatment				post-treatment[b]		
Samples	N_0[a]	CE (%)	10^{-6}xMF	ln(10^6xMF)	N_1[a]	CE (%)	10^{-6}xMF	ln(10^6xMF)
All								
Range	12	2-22	9-137	2.2-4.9	19	1-31	10-161	2.3-5.1
Median		10	29	3.4		7	36	3.6
Mean		11	46	3.5		11	55	3.7
SD		6	38	0.8		9	46	0.8
Excluding those with								

CE<5%	10				14		
Range		7-22	9-73	2.2-4.3	6-31	10-71	2.3-4.3
Median		11	26	3.3	11	32	3.5
Mean		12	35	3.3	14	33	3.4
SD		5	24	0.7	8	18	0.6

[a] $N_0(_t)$ - the number of pre-(post)-treatment samples analyzed
[b] For each patient the means of the values after each treatment cycle were used.

variation was observed (Figure 2.1). The MF increased after treatment in patients 1E, 9E, 17E and 21E, which was accompanied by a decrease in CE (Figure 2.1). There was no effect of etoposide dose on *hprt* MF (lnMF = 3.6 + 0.02 dose, $R^2 = 0.003$, p = 0.8).

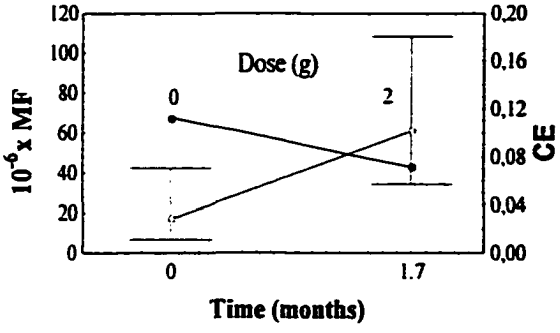
Effects of age and CE on MF. It is well established that MF increases with age by an estimated amount of 1-5% per year in adult donors and that MF is inversely proportional to CE (Cole *et al.*, 1994). Recently, we have analyzed the *hprt* MF by subject age and reported that in subjects over 50 years old the MF does not change with age (Curry *et al.*, submitted). Nevertheless, our patients would rank as "older" and, hence, are expected to be at the high end of the normally observed MFs. The pre-treatment MF in the patients (mean 46±38, range 9-137) is higher than the MF reported for untreated age-matched control population (mean 15±10, range 0.3-39) (Table 2.4). This elevation may not be a reflection of the disease, but rather the fact that the CE in patients (mean 11±6, range 2-22) is much lower than in the control population (mean 38.4±18.6, range 5-92) (Table 2.4). The *hprt* MF in cancer patients before treatment has been reported to be not different from the MF in the age-matched healthy unexposed control population (Branda *et al.*, 1991, Tates *et al.*, 1994, Caggana *et al.*, 1991, Dempsey *et al.*, 1985), however, the

significantly decreased CE has been reported for cancer patients compared to healthy controls (Caggana *et al.*, 1991, Dempsey *et al.*, 1985).

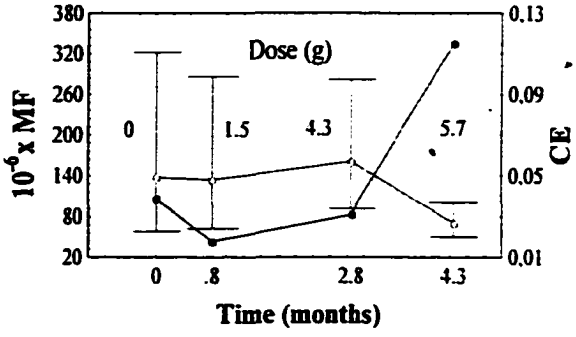
We compared the MF = f (age,CE) relationships obtained in our current study with that determined for the age matched (66-83 years old) unexposed population from the accumulative *hprt* MF data (Table 2.5) (Curry *et al.*, submitted). For both the patients and the age-matched controls, the age had no apparent effect on MF. We did find that MF was inversely related to CE, and though the relationships for both our group and the

Figure 2.1 Results of the *hprt* T-cell cloning assay on CE and MF changes with time in 12 SCLC patients during etoposide chemotherapy. Peripheral blood samples were obtained from the patients before treatment (time 0) and after etoposide cycles (other time points). Pre-stimulated T-cells were grown in 96-well plates in the presence of IL-2 and *hprt* mutants were selected by addition of 6-thioguanine to the medium. The MF for each sample was calculated based on the number of surviving colonies and was adjusted for the corresponding CE. Open circles -MF, closed circles -CE. 95% confidence intervals for MF calculated based on CE and the original number of cells used (Branda *et al.*, 1993 (1)).

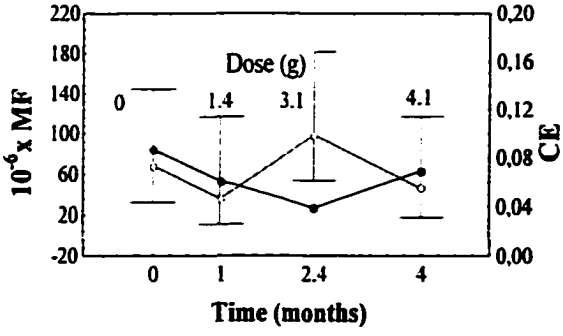
Patient 1E.



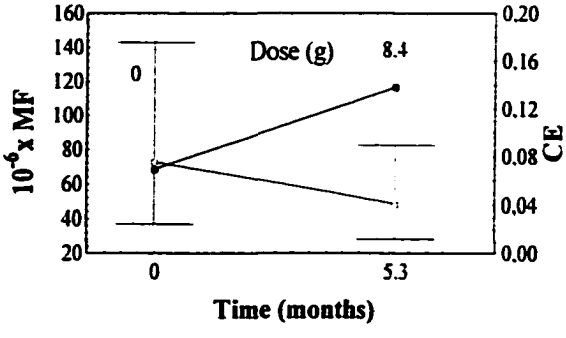
Patient 5E.



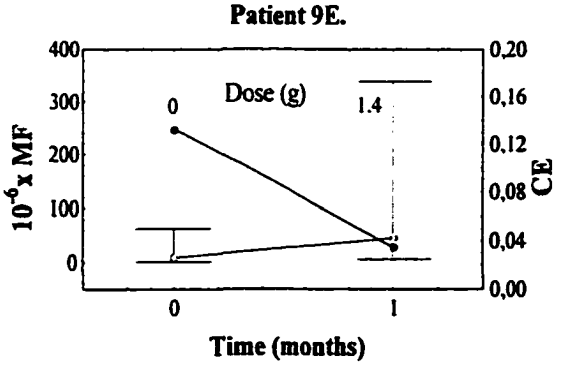
Patient 7E.



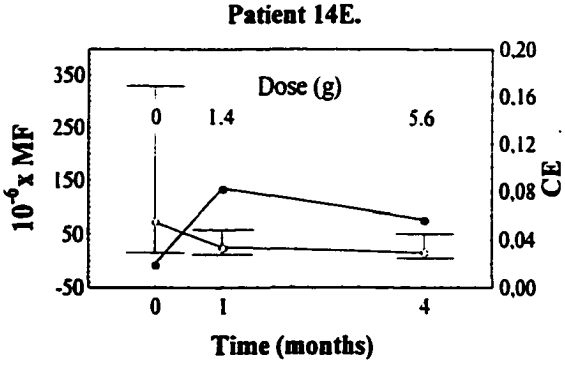
Patient 8E.



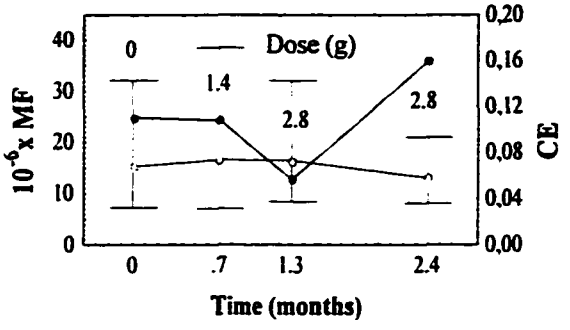
Patient 9E.



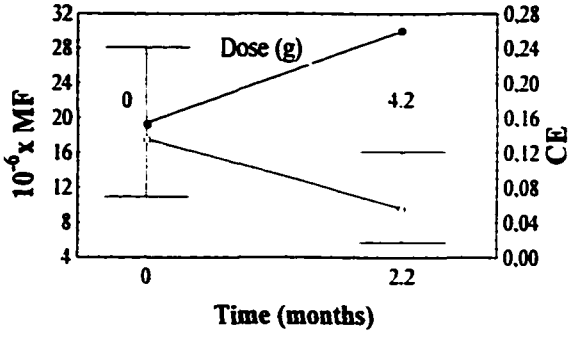
Patient 14E.



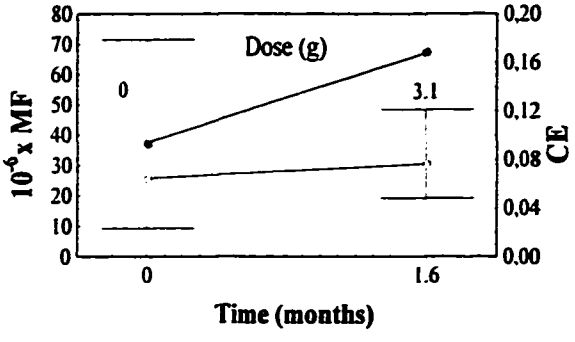
Patient 15E.



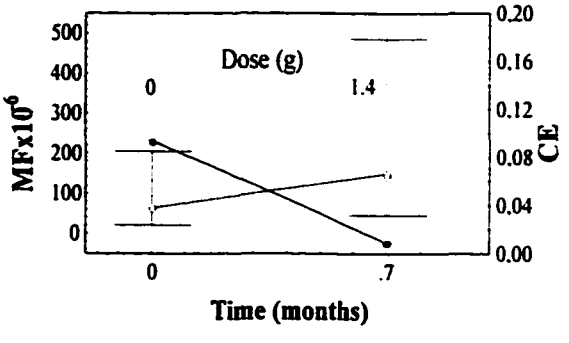
Patient 16E.



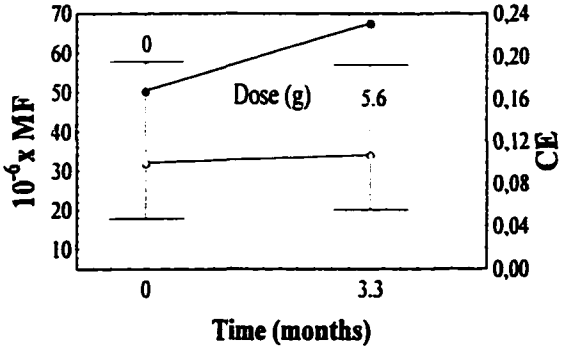
Patient 17E.



Patient 21E.



Patient 22E.



Patient 24E.

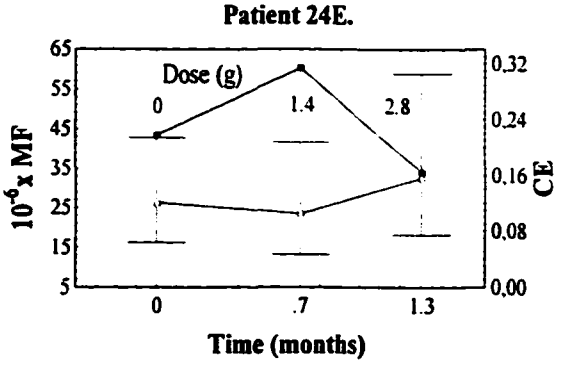


Table 2.4 Results from the previous and current studies on the effect of chemotherapy on mutation measured by the *hprt* T-cell cloning assay in cancer patients[b].

Population/ Cancer type	Treatment	N	Age[a]	pre-treatment CE[a] (%)	pre-treatment MF[a] ($x10^{-6}$)	during and post-treatment CE[a] (%)	during and post-treatment MF[a] ($x10^{-6}$)	Expression time for mutations	Persistence of mutation induction	Ref.
Includes 10 testicular carcinomas	combination chemo	15	36 (16) 16-66	35 (15) 11-62	9 (4) 1-15	39 (18) 7-80	16 (15) 2-100	98± 54 42-172 days	430-490 days	Tates *et al.*, 1994
Solid tumors (9) and lymphomas (18)	combination chemo and/or radiation	27	45 (6) and 55 (3)	UC 21 (17-25)[c] and 15 (11-19)[c]	UC 7 (5-9)[c]	5 (4-7) and 7 (5-8)[c]	20 (8-48)[c] (chemo) 34 (20-60)[c] (chemo+ radiation)		12 months	Dempsey *et al.*,1985
Hodgkin's disease	combination chemo and/or radio	23 and 45	34 (14) and 35 (13) 18-84	UC 11 (10) 2-34 and 21 (18) 1-94	UC 8 (9) 1-41and 4.6 (4) 1-28	11 (14) and 10 (8)	16 (12) and 16 (24)		174 months	Caggana *et al.*, 1991
Breast cancer	radio and/or chemo	17	47 (9)	31 (14)	11 (1)	32 (21)	18 (2)		2 years	Branda *et al.*, 1991
Multiple sclerosis	cyclophosphamide	5	37 (28-50)		4 (Vf)		12-53 (Vf)	2 wks, dropped after 4 wks	normal after 2-3 months	Ammenheuser *et al.*, 1988
ALL and AML	radio and/or combined chemo	45 and 13	12 (5) and 12 (6)	HC 63 (4)	HC 1 (0.2)	59 (4) and 56 (7)	8 (3) and 2 (0.6)		more than 24 months	Hirota *et al.*,1993
Healthy population	none	150	70 (5) 65-85	38 (19) 5-92	15 (10) 0.3-39					Curry *et al.* (submitted)
Our study	Etoposide	12	75 (6) 66-83	11 (6) 2-22	46 (38) 9-137	11 (9) 1-31	55 (46) 10-161			

[a] Mean values, SDs (in parentheses), and ranges.

[b] UC, untreated controls; HC, healthy controls; Vf, variant frequencies in autoradiographic *hprt* assay.

[c] Geometric means and range shown as ± 1 SE.

Table 2.5 Comparison of lnMF = f (age,CE) relationships obtained in the current study with the relationships obtained for the age-matched (66-83 years old) unexposed control population [a].

Source	N	Relationship [b]	R^2 and p values
Curry *et al.*	112	lnMF=1.87 +0.009age	R^2=0.002, p=0.6
(submitted),	113	lnMF=3.20-1.93CE	R^2=0.22, p<0.000001
(CE>5%)	112	CE=0.48-0.0015age	R^2=0.001, p=0.7
Current study.	12	lnMF=3.57-0.0004age	R^2=0.000007, p=1.0
All samples	31	lnMF=4.29-6.05CE	R^2=0.31, p=0.001
	12	CE=0.24-0.0017age	R^2=0.03, p=0.6
Current study.	10	lnMF=5.84-0.03age	R^2=0.09, p=0.4
Samples with	24	lnMF=3.67-2.46CE	R^2=0.07, p=0.2
CE>5%	10	CE=0.08+0.0006age	R^2=0.006, p=0.8

[a] Outliers (cases for which the absolute standard residual value is more than 3) were excluded.
[b] In lnMF=f(age) and CE=f(age) relationships in the current study only values from the pre-treatment samples were included.
N-number of subjects.

database were significant, the slope 6.05 in our group was much higher than the slope 1.93 in the database (Table 2.5). This was largely due to 7 samples, which had a CE of less than 5% and an associated high MF. When these samples were excluded, the slope became lower, but significance also disappeared, probably reflecting the low sample number (Table 2.5). CE was independent of age in our group of patients. This is in agreement with a result from the analysis of CE=f (age) relationship in unexposed population (Table 2.5) (Curry *et al.*, submitted).

Mutational spectra. A total of 425 mutants were recovered from 31 samples including 105 mutants from pre-treatment samples and 320 mutants from post-treatment samples. From 1 to 75 mutants were scored from individual samples (Table 2.2) . A total of 228 mutants were analyzed by sequencing the *hprt* cDNA (Tables 2.6 and 2.7).

Many mutants had structural cDNA alterations such as loss of one or several exons or a part of an exon or insertion of an intron sequence (Tables 2.6 and 2.7). The variant exon splicing in the *hprt* gene can result from mutations at the splice sites, genomic deletions spanning the exon sequence or, sometimes, mutations in the spliced out exons (Steingrimsdottir *et al.*, 1992, Manjanatha *et al.*, 1994). In some cases the underlying mutations in the intron or exon sequence which could result in the structural alterations were identified and these mutants (SC) were included in the spectra with their corresponding mutation type (Tables 2.6 and 2.7). In mutants with mutations affecting splice donor site, the downstream intron sequence was inserted spanning until the next "GT", putative donor site. In mutants where only the beginning part of an exon was missing, the lost sequence usually ended on "AG" sequence, a putative splice acceptor

Table 2.6 List of sequenced *hprt* mutants selected from SCLC patients before etoposide chemotherapy. Mutants were sorted first by the type of mutation and then by their position in the cDNA or by the type of cDNA alteration. Mutants were selected in the *hprt* T-cell clonal assay and the RT-PCR amplified mutant cDNAs were sequenced using ALF automated DNA sequencer. The exact sequence changes, which resulted in aberrant splicing, were determined in some cases by PCR-amplification and sequencing of the appropriate exon/intron region from the genomic DNA.

Mutant no.[a]	Mutation[b]	Position[c]	Exon	Amino acid change[d]	cDNA alteration[e]	Sequence[f]
16E0-4	D 13 bp	341 (342)-353 (354)	4		E 2-3 loss in some	GAC ATA AAA... ATT GGT GGA GAT
15E0-3	D 23 bp	582-604	8			CTT GAC TAT...GAT TTG AAT
24E0-4	D 26676 bp	93-+19	2		565 bp loss	GAT TTG GAA AGG..tgagtttggaaacat
24E0-8	D 6 bp	369 (370,371)-374 (375,376)	4			CTC TCA ACT TTA ACT
7E0-07	F -A	167	3			ATG AAG GAG ATG
1E0-1	F -A	263	3			AAT AGA AAT AGT
16E0-14[g]	F -A	326	4		E 2-3 loss in some	AAT GAC CAG TCA
16E0-21	F -A	523 (524)	7			GGA TAT AAG CCA GAC
1E0-2	SU				17 bp loss from 610 (E9) to 626 (E9)	*CAT GTT....ATT AG*T GAA
24E0-3	SU				17 bp loss from 610 (E9) to 626 (E9)	*CAT GTT....ATT AG*T GAA
5E0-5	SU				5 bp loss from 28 (E2) to 32 (E2), E 2-3 loss in some	*ATT AG*T GAT
14E0-2	SU				5 bp loss from 28 (E2) to 33 (E2)	*ATT AG*T GAT
5E0-1	SU				E 2-3 loss	
17E0-8	SU				E 2-3 loss	

16E0-16	SU				E 2-3 loss	
8E0-7	SU				E 2-3, E 2-6, E 6 loss in some	
24E0-12	SU				E 2-6 loss	
16E0-19	SU				E 4 loss	
16E0-9	SU				E 4 loss	
17E0-4	SU				E 4 loss	
24E0-14	SU				E 4 loss	
8E0-6	SU				E 4 loss	
1E0-4	SU				E 4 loss	
16E0-15	SU				E 4 loss	
5E0-4	SU				E 4 loss in some	
24E0-15	SU				E 4 loss, E 2-4 loss in some or 594 bp loss in some from 28 (E2) to 621 (E9)	***ATT AGT GAT..GTC*** **ATT AGT GAA**
8E0-10	SU				E 5 loss	
24E0-17	SU				E 8 loss	
24E0-7	SU				E 8 loss	
24E0-9	SU				E 8 loss	
8E0-2	SU				E 8 loss	
8E0-4	SU				E 8 loss	
16E0-1	TS A>G	530	7	Asp>Gly		AAG CCA G**A**C Tgtaagt
15E0-6	TS A>G	611	9	His>Arg		ttatagCAT GTT TGT
8E0-11	TS A>G	611	9	His>Arg		ttatagC**A**T GTTTGT
24E0-1[h]	TS C>T	508	7	Arg>stop		CCA **C**GA AGT GTT
24E0-18[h]	TS C>T	508	7	Arg>stop		CCA **C**GA AGT GTT
5E0-7	TS C>T	508	7	Arg>stop		ACC CCA **C**GA AGT GTT
1E0-3	TS G>A	27	1	no change		GTC GT**G**gtgagc
1E0-5	TS G>A	173	3	Gly->Glu		ATG G**G**A GGC

22E0-5	TS G>A	197	3	Cys>Tyr		GCC CTC TGT GTG CTC
24E0-6	TS G>A	197	3	Cys>Tyr		GCC CTC TGT GTG CTC
16E0-18[h]	TS G>A	400	5	Glu>Lys		GTG GAAgtaagttca
16E0-8[h]	TS G>A	400	5	Glu>Lys		GTG GAAgtaagttca
24E0-2[g]	TS G>A	509	7	Arg>Gln	E 2-6 loss	ACC CCA CGA AGT GTT
9E0-1	TS G>A	617	9	Cys>Tyr		CAT GTT TGT GTC ATT
17E0-2	TV A>C	116	2	His>Pro		ATT CCT CAT GGA CTA
7E0-05	TV A>C	116	2	His>Pro		ATT CCT CAT GGA
16E0-22	TV C>G	551	8	Pro>Ala		GAA ATT CCA GAC AAG
16E0-17	TV G>C	3	1	Met>Ile		tccgttATG GCG ACC
24E0-19	TV G>C	208	3	Gly>Arg		CTC AAG GGG GGC
16E0-5	TV G>C SC	+ 5(I5)			IS 67 bp from +1(I5) to +67 (I5)	GTG GAA*gtaagttca...atcctaaag*gtaagcc
17E0-3	TV G>T	11	1	Arg>Leu		GCG ACC CGC AGC CCT
14E0-1	TV G>T	47	2	Gly>Ala		GAA CCA GGT TAT GAC
24E0-20	TV G>T	143	3	Arg>Leu		GAA CGT CTT GCT
8E0-8	TV T>A	104	2	no change		GAA AGG GTG TTT ATT
16E0-3	TV T>G	23	1	Val>Asp		GGC GTC GTG
5E0-3	TV T>G	198	3	Cys>Trp		GCC CTC TGT GTG CTC

[a] Mutants were identified according to the patient they were obtained from and time relative to treatment. For example, 1E0-1 refers to mutant number 1 obtained from patient 1E before treatment ("0" refers to pre-treatment sample). Mutants obtained from split and extra samples not included in MF data (Table 2.3 and Figure 2.1) have alternative names, for example, 7E0-07, 7E0-05.

[b] Mutations are classified as follows: D-deletion; T-tandem mutation; F-frameshift (+1 insertion or -1 deletion); SU (SC) - alternatively cDNA splicing with unidentified (characterized) underlying mutation; C-complex mutation; TS-transition; TV-transversion; NF- no mutation could be found.

[c,e] Base number corresponding to the mutation is given (base 1 is an "A" of AUG initiation codon (Edwards *et al.*, 1990)). Intron sequences (I) are numbered indicating the intron number in brackets and are either with "+", where "+1" is the first base pair following the exon or "-", where "-1" is the first base pair preceding the exon. Genomic sequence before the initiating codon or after the stop codon is numbered in a similar manner only without the intron number in brackets. For + 1 frameshifts the position of the preceding base is indicated and apostrophe indicates insertion of the base. Base pairs in brackets indicate alternative positions for mutation, an uncertainty due to the repetitive sequences at the ends of deletions and insertions.

[d] "Stop"- stop codon.

[e] Mutants with structural alterations in cDNA such as the whole or partial exon loss and insertion (IS) of intron sequence are shown. In majority of cases the analysis of underlying mutation was not done and such mutants were characterized as having unidentified splice errors (see text). E - exon; I- intron.
[f] The sequence context for mutations and cDNA alterations is given. Mutated bases are underlined, repeat and complement sequences are shown in bold, intron sequences are in small cases and inserted sequences are shown in italics. The lost bases in unidentified splice mutants (SU) are italicized and underlined.
[g] Mutants with an identical mutation obtained from the same sample.
[h] Mutants with exon losses that were additional to the point mutations outside of the missing exons.

Table 2.7 List of sequenced *hprt* mutants selected from SCLC patients after etoposide chemotherapy. Mutants were sorted first by the type of mutation and then by their position in the cDNA or by the type of cDNA alteration. Mutants were selected in the *hprt* T-cell clonal assay and the RT-PCR amplified mutant cDNAs were sequenced using ALF automated DNA sequencer. The exact sequence changes which resulted in aberrant splicing were determined in some cases by PCR-amplification and sequencing of the appropriate exon/intron region from the genomic DNA.

Mutant no.[a]	Mutation[b]	Position[c]	Exon	Amino acid change[d]	cDNA alteration[e]	Sequence[f]
17E2-2	D 34 bp SC	14 -+ 20 (I1)	1		IS 29 bp from +21 (I1) to + 49 (I1)	A**GC CCT GGC** GTC GTGgtg...tgccg*gccctggccg..cag*gtggcg
7E3-19	C T>AC	23	1			A**TG GCG AC**C CGC...CC**T GGC GTC** GTG
5E3-10	D 11 bp	42 (43,44)-52 (53,54)	2			GAT GAT GA**A CCA...TAT GAC CTT** GAT
22E4-8[g]	D 11 bp	116-126	2			CCT C**AT GGA CTA ATT ATG GAC** Aggt
22E4-4[g]	D 11 bp	116-126	2			CCT C**AT GGA CTA ATT ATG GAC** Aggt
22E4-15[g]	D 11 bp	116-126	2			CCT C**AT GGA CTA ATT ATG GAC** Aggt
22E4-9[g]	D 11 bp	116-126	2			CCT C**AT GGA CTA ATT ATG GAC** Aggt
22E4-13[g]	D 11 bp	116-126	2			CCT C**AT GGA CTA ATT ATG GAC** Aggt
22E4-10[g]	D 11 bp	116-126	2			CCT C**AT GGA CTA ATT ATG GAC** Aggt
22E4-1[g]	D 11 bp	116-126	2			CCT C**AT GGA CTA ATT ATG GAC** Aggt
22E4-14[g]	D 11 bp	116-126	2			CCT C**AT GGA CTA ATT ATG GAC** Aggt
15E2-2	D 16 bp SC	-5-11	1		11 bp loss from 1(E1) to 11 (E1)	ccggctccgtt ATG...CGC AGC CCT GGC
8E6-6	D 20 bp SC	+ 5 (I1)-+ 24 (I1)			IS 29 bp from +1 (I1)-+4 (I1) to +25 (I1)-+49 (I1)	GTG*gtgagcagct..ggccctggccggtt..ggcag*gtgg
8E6-1	D 26 bp	578-603	8			GCC C**TT GAC...AGG GAT TTG AA**Tgtaagt
16E3-4	D 26 bp	281-306	3			TCC **ATT C**CT ATG....AGA CTG **AAG** AGC
15E2-11	D 3 bp	124-126	2			**AT GGA CTA ATT ATG GAC**

22E4-3	D 3 bp	623-625	9		TGT GTC ATT AGT GAA
15E2-8	D 37 bp SC	-17 (-16,-15) to 20 (21, 22)	1	20 bp loss from 1 (E1) to 20 (21, 22) (E1)	cagtcagcccgcgcgccg...ATG...C AGC CCT GGC GTC
5E4-32	D 8 bp	56 (57,58)-63 (64,65)	2		GAC CTT GAT TTA TTT TGC ATA
5E1-4	D 9 bp	41 (42)-49 (50)	2	in some 522 bp loss from (54-60) (E2) to (575-581) (E8)	GAT GAA CCA...TAT GAC CTT GAT, GAC CTT GAT...TAT GCC CTT GAC
17E2-25	D 9 bp	290-298	3		ATG ACT GTA GAT TTT ATC AGA
1E2-6	F +T	531'	7		CCA GAC T'gtaagtgaa
15E1-5	F +A	7	1		ATG GCG A'CC CGC AGC
5E4-21	F +A	(421-424)'	6		ACT GGC AAA A'CA
5E4-48	F -A	(98-100)	3		GAT TTG GAA AGG GTG
16E3-2	F -C	610	9		tatag CAT GTT TGT GTC
22E4-5	F -T	75	2		TGC ATA CCT AAT CAT TAT
15E2-10	F -A	607(608)	8		T TTG AAT gtaagtaat
15E2-4	F -G	538(539)	8		tttttag TT GTT GGA TTT GAA
14E1-5	NF				
15E2-9	NF				
17E2-21	NF				
22E4-7	NF				
5E3-1	NF				
5E3-34	NF				
5E4-1	NF				
5E4-25	NF				
5E4-34	NF				
5E4-51	NF				
5E4-53	NF				
5E4-73	NF				

8E6-9	NF		
7E3-31	SU	21 bp loss from 533 (E8) to 553 (E8)	*TT GTT ...ATT CCA G*AC AAG
14E4-1	SU	5 bp loss from 28 (E2) to 33 (E2)	*ATT AG*T GAT
5E4-11	SU	E 2-3 and 6 loss in some	
5E04-50	SU	E 2-3 loss	
17E2-1	SU	E 2-3 loss	
24E1-10	SU	E 2-3 loss	
24E2-13	SU	E 2-3 loss	
5E4-10	SU	E 2-3 loss	
5E4-8	SU	E 2-3 loss	
5E3-23	SU	E 2-3 loss	
5E3-5	SU	E 2-3 loss	
8E6-2	SU	E 2-3 loss	
9E1-1	SU	E 2-3 loss	
5E1-16	SU	E 2-3 loss in some	
5E3-9	SU	E 2-3 loss in some	
5E4-26	SU	E 2-3 loss in some	
8E6-3	SU	E 2-3 loss in some	
8E6-5	SU	E 2-3 loss in some	
5E4-61	SU	E 2-4 loss in some	
5E04-4	SU	E 2-6 loss	
14E4-2	SU	E 2-6 loss in some	
16E3-3	SU	E 2 loss	
5E4-29	SU	E 2 loss	
1E2-10	SU	E 2 or 3 or 2-3 loss	
16E3-19	SU	E 4 loss	
24E1-7	SU	E 4 loss	

1E2-7	SU	E 4 loss
1E2-8	SU	E 4 loss
5E3-24	SU	E 4 loss in some
5E4-12	SU	E 5 loss
5E3-15	SU	E 5 loss
5E3-33	SU	E 5 loss
5E4-5	SU	E 5 loss
7E 04-4	SU	E 5 loss
7E3-32	SU	E 5 loss
7E4-4	SU	E 5 loss
7E3-23	SU	E 5 loss, E 2-3 or 2-6 loss in some
7E3-02	SU	E 5 loss, E 6 loss in some
7E 04-19	SU	E 6 loss
5E1-13	SU	E 6 loss, E 2-6 loss in some
15E2-1	SU	E 7 loss
15E2-7	SU	E 7 loss
17E2-10	SU	E 7 loss
1E2-9	SU	E 7 loss
5E4-23	SU	E 7 loss, E 2-7 loss in some
15E1-2	SU	E 8 loss
5E1-10	SU	E 8 loss
5E1-17	SU	E 8 loss
7E 04-16	SU	E 8 loss
7E 04-2	SU	E 8 loss
7E4--10	SU	E 8 loss
5E3-14	SU	E 8 loss in some

5E3-8	SU				E 8 loss in some	
7E4--15	SU				E 8 loss, E 2-3 and 6 loss in some	
24E2-1	SU				E 8 loss, E 2-3 loss in some	
5E4-7	SU				1 bp loss 402 (E5), 50 bp loss from 403 (E6) to 452 (E6)	GTG GAΔgtaa..gaaag*GAT..GTC AG*G CAG
16E3-16	T D 62 bp, D 26 bp	423-484, 536-561	6,8			GGC AAA ACA....C GCA A Ggtatg, ag TT GTT GGA.....aag TTT GTT GTA
24E2-9	T CC>TT	463,464	6	Pro>Leu		TAT AAT CCA AAG ATG
5E4-55	TS A>G	404	6	Asp>Gly		tttgaaagGAT ATA ATT
5E3-3	TS A>G	602	8	Asp>Gly		TTC AGG GAT TTG AAT
17E2-13	TS A>G	611	9	His>Agr		ttatagCAT GTT TGT
8E6-13	TS A>G	611	9	His>Arg		ttatagCAT GTTTGT
14E4-3	TS C>T	145	3	Leu>Phe		GAA CGT CTT GCT CGA
16E3-15	TS C>T	151	3	Arg>Gly		GCT CGA GAT
5E1-1	TS C>T	464	6	Pro>Leu		TAT AAT CCA AAG ATG
5E1-5	TS C>T	610	9	His>Asp		tttttatagCAT GTT TGT
5E4-63	TS C>T	463	6	Pro>Ser	in some 583 bp loss from 64 (E2) to 646 (E9)	AAT CCA AAG, GAT TTA TTT TGC ATA.. GGA AAA GCA AAA TAC AAA
5E3-21	TS G>A	118	2	Gly>Arg		CCT CAT GGA CTA ATT ATG
14E1-7	TS G>A	143	3	Arg>His		ACT GAA CGT CTT GCT
16E3-13	TS G>A	143	3	Arg>His		ACT GAA CGT CTT GCT
17E2-22	TS G>A	143	3	Arg>His		ACT GAA CGT CTT GCT
5E4-9	TS G>A	168	3	no change		ATG AAG GAG ATG GGA
17E2-11	TS G>A	197	3	Cys>Tyr		GCC CTC TGT GTG CTC
5E4-6	TS G>A	197	3	Cys>Tyr		GCC CTC TGT GTG CTC AAG
15E1-6	TS G>A	209	3	Gly>Glu		CTC AAG GGG GGC

5E3-37	TS G>A	209	3	Gly>Glu		AAG GGG GGC
1E2-3	TS G>A	212	3	Gly->Asp		AAG GGG GGC TAT
7E3-04	TS G>A	539	8	Gly->Glu		ttagTT GTT GGA TTT GAA
17E2-3	TS G>A	580	8	Asp>Asn		GCC CTT GAC TAT AAT
5E3-29[h]	TS G>A	606	8	Leu>Leu	E 2-6 loss	GAT TTG AATgtaagtaa
1E2-5	TS G>A	606	8	no change		AGG GAT TTG AATgtaagta
17E2-17[h]	TS G>A	617	9	Cys>Tyr	E 2-3 loss	CAT GTT TGT GTC ATT
24E2-10	TS G>A	617	9	Cys>Tyr		CAT GTT TGT GTC
5E3-30	TS G>A	617	9	Cys>Tyr		CAT GTT TGT GTC ATT
5E4-24[g]	TS G>A	617	9	Cys>Tyr		CAT GTT TGT GTC ATT
5E4-72[g]	TS G>A	617	9	Cys>Tyr		CAT GTT TGT GTC ATT
8E6-14[g]	TS G>A	617	9	Cys>Tyr		CAT GTT TGT GTC ATT
8E6-8[g]	TS G>A	617	9	Cys>Tyr		CAT GTT TGT GTC ATT
5E4-42[h]	TS G>A	322	4	Asp>Asn	E 2-3 loss	AAT GAC CAG TCA
7E3-20	TS G>A SC	544	8		E 8 loss in some	GGA TTT GAA ATT
7E4-20	TS G>A SC	- 1(I7)			E 8 loss or 21 bp loss from 533 (E8) to 553 (E8)	ctttttag*TT GTT ...ATT CCA G*AC AAG
14E1-2	TS G>A SC	+ 1(I1)			IS 49 bp from +1 (I1) to +49 (I1)	GTG*gtgagcagct...ggcag*gtgg
8E6-11	TS G>A SC	+ 5(I1)			IS 49 bp from +1 (I1) to +49 (I1)	GTG*gtgagcagct...ggcag*gtgg
5E3-11	TV A>T	109	2	Ile>Phe		GTG TTT ATT CCT
1E2-1	TV A>T	131	2	Asp->Val		ATT ATG GAC Aggtaa
16E3-17[g]	TV A>T	133	2	Arg>Trp		ATG GAC AG gtaagtaag
16E3-18[g]	TV A>T	133	2	Arg>Trp		ATG GAC AG gtaagtaag
22E4-12	TV A>T	133	2	Arg>Trp		ATG GAC AGgtaagtaag
5E4-4	TV A>T	133	2	Arg>Trp		ATG GAC AGgtaagtaa
5E3-28	TV A>T	404	6	Ile>Leu		GAT ATA ATT
5E4-35	TV A>T	581	8	Asp>Val		GCC CTT GAC TAT AAT

5E4-58	TV A>T	602	8	Asp>Val		TTC AGG GAT TTG AAT
7E3-11	TV A>T	611	9	His->Leu		ttatagCAT GTT TGT
15E2-12	TV A>T	637	9	Lys>stop		GGA AAA GCA
7E4-7[h]	TV A>T	642	9	no change	E 2-3 loss in some	AAA GCA AAA TAC
22E4-2	TV C>A	550	8	Pro>Thr		GAA ATT CCA GAC AAG
5E1-14	TV C>G	115	2	His>Asp		ATT CCT CAT GGA CTA
8E6-17	TV C>G	368	4	Ser>stop		GAT CTC TCA ACT TTA
7E 04-8	TV G>C	3	1	Met->Ile		tccgttATG GCG
7E4-13	TV G>C	135	3	Arg->Ser		ctgtagG ACT GAA
14E1-1	TV G>C	152	3	Arg>Pro		CTT GCT CGA GAT GTG
16E3-14	TV G>C	606	8	Leu>Phe		AGG GAT TTG AAT gtaagtaat
16E3-7	TV G>C SC	96	2	Leu>Phe	Ex 2-3 loss in some	GAC GAT TTG GAA AGG AGG
5E3-19	TV G>T	119	2	Gly>Val		CCT CAT GGA CTA ATT ATG
5E1-12	TV T>A	2	1	Met>Lys		tccgttATG GCG ACC
5E3-12	TV T>A	2	1	Met>Lys		tccgttATG GCG
16E3-1	TV T>A	84	2	Tyr>stop		CAT TAT GCT
1E2-4	TV T>A	158	3	Val->Glu		CGA GAT GTG ATG
14E1-3	TV T>A	158	3	Val>Glu		CGA GAT GTG ATG AAG
5E1-7	TV T>A	389	5	Val>Asp		AAT GTC TTG ATT
15E2-3	TV T>A	542	8	Phe>Tyr		GTT GGA TTT GAA ATT
8E6-15	TV T>G	29	2	Ile>Ser		ttcagATT AGT GAT
22E4-16	TV T>G	194	3	Leu>Arg		GTA GCC CTC TGT GTG
7E3-12	TV T>G	203	3	Leu->Arg		TGT GTG CTC AAG
1E2-2	TV T>G	449	6	Val->Gly		TCC TTG GTC AGG
7E3-13	TV T>G	571	8	Tyr->Asp		GTA GGA TAT GCC
5E4-18	TV T>G	595	8	Phe>Leu		GAA TAC TTC AGG GAT
15E2-6	TV T>G	488	7	Leu>Gly		aacag C TTG CTG GTG

[a] Mutants were identified according to the patient they were obtained from and time relative to treatment. For example, 24E2-10 refers to mutant number 10 obtained from patient 24E after 2 etoposide cycles ("2" refers to the number of post- treatment cycles). Mutants obtained from split and extra samples not included in MF data (Table 2.3 and Figure 2.1) have alternative names, for example, 5E04-50, 7E4--10, and 7E3-02.

[b] Mutations are classified as follows: D-deletion; T-tandem mutation; F-frameshift (+1 insertion or -1 deletion); SU (SC) - alternatively cDNA splicing with unidentified (characterized) underlying mutation; C-complex mutation; TS-transition; TV-transversion; NF- no mutation could be found.

[c,e] Base number corresponding to the mutation is given (base 1 is an "A" of AUG initiation codon (Edwards *et al.*, 1990)). Intron sequences (I) are numbered indicating the intron number in brackets and are either with "+", where "+1" is the first base pair following the exon or "-", where "-1" is the first base pair preceding the exon. Genomic sequence before the initiating codon or after the stop codon is numbered in a similar manner only without the intron number in brackets. For + 1 frameshifts the position of the preceding base is indicated and apostrophe indicates insertion of the base. Base pairs in brackets indicate alternative positions for mutation, an uncertainty due to the repetitive sequences at the ends of deletions and insertions.

[d] "Stop"- stop codon.

[e] Mutants with structural alterations in cDNA such as the whole or partial exon loss and insertion (IS) of intron sequence are shown. In majority of cases the analysis of underlying mutation was not done and such mutants were characterized as having unidentified splice errors (see text). E - exon; I- intron.

[f] The sequence context for mutations and cDNA alterations is given. Mutated bases are underlined, repeat and complement sequences are shown in bold, intron sequences are in small cases and inserted sequences are shown in italics. The lost bases in unidentified splice mutants (SU) are italicized and underlined.

[g] Mutants with an identical mutation obtained from the same sample.

[h] Mutants with exon losses that were additional to the point mutations outside of the missing exons.

site, implicating that the mutation affected the actual splice acceptor site in the preceding intron (Tables 2.6 and 2.7). In the majority of exon loss mutants (SU) the underlying mutation has not been determined (Tables 2.6 and 2.7). These include identical exon loss mutants obtained from the same sample or from different samples but from the same patient. Although the possibility of their clonality can not be excluded, these mutants are likely independent and were considered so for this analysis. Part of the reasoning is that many different mutations can result in the same splice error and the clonal independence of such mutants has been demonstrated previously (Curry *et al.*, 1995).

In six mutants 2-3 and 2-6 exon losses were detected together with a point mutation outside the lost exons (Tables 2.6 and 2.7). These mutants were classified according to their point mutation (G>A transition in 4 cases, one -A frameshift and one A>T transversion that did not change amino acid). However, because the sequence analysis within the intron portion of the splice sites was not done, the possibility of additional mutations at the splice sites can not be excluded.

Interestingly, in many cases of splice mutants multiple cDNA products were obtained after RT-PCR amplification as evidenced by the presence of multiple bands on the agarose gel (data not shown). These multiple products indicate the presence of various cDNA populations in RT-PCR product and, thus, certain cDNA alterations can be applicable only to some proportion of the total number of cDNA molecules in the product (Tables 2.6 and 2.7, column 6). Multiple cDNA species after RT-PCR amplification of *hprt* mRNA have been reported in other studies where nonsense mutations were for the most part responsible for the variant exon splicing (Steingrimsdottir *et al.*, 1992, Manjanatha *et al.*, 1994, Curry *et al.*, 1995). In our study it was not possible to fully

analyze the types of mutations which led to the production of multiple cDNA species, because the underlying mutations were not identified in the majority of splice mutants. It is worth mentioning, however, that the proportion of various cDNA populations and the appearance of multiple bands in PCR products varied when the same mutant was analyzed several times with different cell pellets in the same RT-PCR reaction. This indicates that the composition of the cell pellet and the individual RT-PCR reaction may influence the production of multiple cDNA species.

There were two mutants (5E1-4 and 5E4-63) which had more than 500 bp loss from a portion of their cDNA molecules on top of identified mutations outside of the lost regions (deletion of 9 bp and G>A transition respectively). These mutants were classified according to their mutations, which possibly could have indirectly caused the occurrence of the losses of several exons in some cDNA molecules. Interestingly, the lost regions were flanked by 6 bp direct repeats in 5E1-4 mutant and by 8 bp complement sequences with a potential for hairpin-loop structure formation in mutant 5E4-63. Interestingly, together with another mutant, 24E0-15, these three mutants had similar cDNA losses from the middle of exon 2 to the various sequences inside exons 8 or 9.

In one sample, 22E-4, 8 out of 14 mutants were found to bear the same 11 bp deletion in exon 2 flanked by 6 bp repeats. Five other samples, 16E-0, 24E-0, 5E-4, 8E-6, and 16E-3 each had a couple of mutants with identical base pair substitutions. In each case only one of the identical mutants was included in the spectra analysis. Also, the analysis did not include 13 mutants where no mutation was identified.

Spectra comparisons. A total of 56 pre-treatment and 147 post-treatment independent mutations were identified and the spectra of mutations were compared (Table 2.8). Both

Table 2.8 Comparison of spectra of mutations found in unexposed control population (Curry *et al.*, submitted), and in SCLC patients before and after etoposide chemotherapy.

MUTATION CLASS	UNEXPOSED CONTROL		PRE-ETOPOSIDE		POST-ETOPOSIDE	
GC > AT	45	13.4%	9	16.1%	25	17.0%
AT > GC	15	4.5%	3	5.4%	4	2.7%
Transitions	60	17.8%	12	21.4%	29	19.7%
GC > TA [c]	**12**	**3.6%**	**3**	**5.4%**	**2**	**1.4%**
GC > CG	22	6.5%	3	5.3%	6	4.1%
AT > TA [b]	**22**	**6.5%**	**1**	**1.8%**	**18**	**12.2%**
AT > CG	17	5.0%	4	7.1%	7	4.8%
Transversions	73	21.7%	11	19.6%	33	22.4%
TOTAL BP CHANGES	133	39.5%	23	41.1%	62	42.2%
Frameshifts - 1bp	25	7.4%	4	7.1%	5	3.4%
Frameshift + 1bp	5	1.5%	0	0	3	2.0%
Splice errors [a]	129	38.3%	25	44.6%	65	44.2%
Deletions	29	8.6%	4	7.1%	9	6.1%
Complex	9	2.7%	0	0	1	0.7%
Insertions	4	1.2%	0	0	0	0
Tandem	3	0.9%	0	0	2	1.4%
TOTAL	337	100%	56	100%	147	100%

[a] Splice errors include characterized and uncharacterized mutations which led to variant splice events.

[b] The proportion of AT >TA transversions was different between the pre-etoposide (1 out of 56 total mutations) and post-etoposide (18 out of 147 total mutations) spectra with $\chi^2=4.3$ and $p<0.037$

[c] The proportion of GC>TA transversions was different between the pre-etoposide (3 out of 56 total mutations) and post-etoposide (2 out of 147 total mutations) spectra with $\chi^2=5.0$ and $p<0.025$

spectra were also compared to the control spectrum from unexposed individuals collected in the *hprt* database (MutaBase software, Durham, NC) and analyzed by us (Table 2.8) (Curry *et al.*, submitted). The spectra were found to be similar with the most frequent mutations being base pair substitutions (39.5%, 41.1% and 42.2% as in control pre- and post-etoposide spectra respectively), mutations leading to splice errors such as exon skipping events (38.3%, 44.6%, 44.2%), followed by deletions (8.6, 7.1, and 6.1%), and +/- frameshifts (8.9%, 7.1% and 5.4%).

The analysis of distribution of mutations affecting exon splicing revealed differences among spectra (Table 2.9). In unexposed control population exons 2 (29%), 8 (26%) and 4 (14%) were affected the most. Exons 2 and 8 continued to be often affected in pre-etoposide (28% and 20% respectively) and post-etoposide (37% and 20% respectively) mutation spectra, however, mutations affecting splicing of exon 4 was significantly under-represented in post-etoposide (8%) compared to pre-etoposide (36%) spectra, where it was significantly over-represented compared to untreated control spectra (14%). At the same time, exon 1 was significantly more often involved in aberrant splicing in post-etoposide spectrum (9%) compared to unexposed control (1%). Exon 5 was also over-represented in aberrant splicing events in post-etoposide (15%) compared to unexposed control (7%) spectrum. There was no splice mutations recovered affecting exon 9 in post-treatment mutants, that is significantly different from 6% recovery in unexposed control population.

Four and thirteen deletion mutations were recovered from pre- and post-treatment mutants respectively (Table 2.10). The size of deletion ranged generally from 3 to 37 bp, however, in one pre-treatment mutant (24E0-4) a large deletion of 26,676 bp was found

Table 2.9 Distribution of mutations affecting exon splicing in the *hprt* gene in control unexposed population (Curry *et al.*, submitted) and SCLC patients before and after etoposide treatment[a,b,c].

Exon	Unexposed control		Pre-etoposide		Post-etoposide	
1	1	1%	0	0	6	9%
2	38	29%	7	28%	24	37%
3	3	2%	0	0	0	0
4	18	14%	9	36%	5	8%
5	9	7%	2	8%	10	15%
6	10	8%	0	0	2	3%
7	9	7%	0	0	5	8%
8	33	26%	5	20%	13	20%
9	8	6%	2	8%	0	0
Total splice	129	100%	25	100%	65	100%

[a] Mutations affecting the splicing of several exons were included in the certain class according to the most 5' exon being spliced out.

[b] Exon 4: unexposed control *vs.*. pre-etoposide, χ^2=7.3, p<0.006.
Exon 4: pre-etoposide *vs.* post-etoposide, χ^2=8.75, p<0.003.
Exon 1: unexposed control *vs.* post-etoposide, χ^2=11.2, p<0.0008.
Exon 5: unexposed control *vs.* post-etoposide, χ^2=3.9, p<0.04.
Exon 9: unexposed control *vs.* post-treatment, χ^2=4.8, p<0.02

[c] The spectra include exon skipping mutants with identified or unidentified underlying mutation.

Table 2.10 The distribution of deletion mutations in the *hprt* gene in the control unexposed population (Curry *et al.*, submitted) and in SCLC patients before and after etoposide chemotherapy[a,b].

Exon (E) / Intron (I)	Unexposed control		Pre-etoposide		Post-etoposide	
1	0	0				
2	10	30%			5	39%
3	8	24%			2	15%
4	2	6%	2	50%		
5	0	0				
6	4	12%				
7	1	3%				
8	3	9%	1	25%	1	8%
9	1	3%			1	8%
-,E1					2	16%
E1,I1	1	3%			1	8%
I1					1	8%
I2,E3	2	6%				
E2,+			1	25%		
I7	1	3%				
Total	33		4		13	
Deletion size range (bp)	2-66		6-23, 584		3-37	

[a] -/+ - Before/after stop codon, deletions spanning exon/intron junctions are indicated with the positions of both breakpoints.

[b] The spectra include identified deletion mutations, which led to aberrant exon splicing.

(Table 2.6). Three out of four (75%) deletion mutations from pre-treatment mutants were immediately flanked by 4 or 8 bp direct repeats suggesting the misalignment mechanism in their formation, whereas five out of thirteen (38%) deletion mutants from post-treatment mutants were flanked by 3-8 bp immediate direct repeats, three mutations had distal 3-6 bp direct repeats, two had immediate flanking complement 3 bp sequences (two mutants with deletions in the same region of exon 3), one had 6 bp imperfect direct repeat and two had no repeats (Tables 2.6 and 2.7).

The distribution of deletion mutations was similar between unexposed control and post-etoposide spectra in the proportion of deletions concentrated mostly in exon 2 (30% and 39% respectively) and exon 3 (24% and 15% respectively) (Table 2.10). No deletions in exon 6, the next most common exon for this type of mutation in unexposed control spectra (12%), were recovered neither in pre- nor post-treatment spectra. Interesting, however, was the finding of 4 deletion mutations with breakpoints clustered in exon 1 boundaries and intron 1 in post-treatment spectrum (Tables 2.7 and 2.10). All four mutations led to the aberrant splicing of exon 1 in the *hprt* cDNA (Table 2.7). These deletions were responsible for the increase in the exon 1 splice errors in post-etoposide spectrum (Table 2.9). Only one deletion mutation, leading to aberrant splicing of exon 1 has been recorded in the unexposed control spectra of 33 deletion mutations (Table 2.10).

Changes in spectra were seen in the frequency of different classes of base substitutions (Table 2.11). Although no significant difference was seen between the pre-etoposide and control spectra, the post-etoposide spectra differed significantly from both previous spectra in the proportion of GC>TA and AT>TA transversions. The contribution of GC>TA transversions decreased (from 12.3% and 12.5% to 3.0%) while the number of

Table 2.11. Comparison of spectra of base substitution mutations from the control unexposed population (Curry *et al.*, submitted)[c] and SCLC patients before and after etoposide chemotherapy.

MUTATION CLASS	UNEXPOSED		PRE-ETOPOSIDE		POST -ETOPOSIDE	
GC>AT	93	36.5%	9	37.5%	29	43.3%
AT>GC	31	12.2%	3	12.5%	4	6.0%
Transitions	124	48.6%	12	50%	33	49.2%
GC>TA [b]	**32**	**12.5%**	**3**	**12.5%**	**2**	**3.0%**
GC>CG	36	14.1%	4	16.7%	7	10.4%
AT>TA [a]	**35**	**13.7%**	**1**	**4.2%**	**18**	**26.9%**
AT>CG	28	11.0%	4	16.7%	7	10.4%
Transversions	13	51.4%	12	50%	34	50.7%
Total bp changes	255	100%	24	100%	67	100%

[c] Control spectrum includes additional base substitutions reported by Burkhart-Schultz *et al.* (1996) (only point mutations were reported in the paper) which were not included in the whole spectra in Table 8. Three spectra also include base substitutions, which led to splice errors in characterized splice mutants.

[a] The proportion of AT>TA transversions was different between the control (35 out of 255 total mutations) and post-etoposide (18 out of 67 total mutations) spectra with χ^2=5.6 and p<0.02 and between the pre-etoposide (1 out of 24 total mutations) and post-etoposide (18 out of 67 total mutations) spectra with χ^2=4.3 and p<0.04.

[b] The proportion of GC>TA transversions was different between the control (32 out of 255 total mutations) and post-etoposide (2 out of 67 total mutations) spectra with χ^2=4.5 and p<0.03 and between the pre-etoposide (3 out of 24 total mutations) and post-etoposide (2 out of 67 total mutations) spectra with χ^2=5.0 and p<0.03.
of 255 base substitution mutations from unexposed individuals (Curry *et al.*, submitted).

AT>TA transversions increased (from 13.7% and 4.2% to 26.9%) (Table 2.11). These AT>TA transversions in post-etoposide spectra were recovered from different patients (7 out of 12) with similar frequency (9.5%-20% of the total number of sequenced mutants from individual patients). Interestingly, three out of 18 AT>TA transversions occurred at position 133 of exon 2. Only one such mutation at this position has been reported in the database with 35 AT>TA transversions. Also, out of 14 positions found to have AT>TA transversion in post-etoposide spectra only 4 had this mutation recovered in the unexposed control spectra (Table 2.7) (Curry *et al.*, submitted).

The exon distribution of base substitutions was similar in unexposed control population and in post-etoposide spectra with the only difference reaching the borderline significance (χ^2=4.1, p<0.04) being the reduction in the proportion of base substitutions in exon 7 (1 out of 67 *vs.* 22 out of 255) in post-etoposide spectrum compared to untreated control spectrum (data not shown). The number of GC>AT transversions at CpG sites were similar in the unexposed control spectrum (14 out of 93 GC>AT transversions or 15%) and post-etoposide spectrum (4 out of 29 GC>AT transversions or 14%). CpG sites at ositions 143, 151, 508 were mutated in both spectra. This type of mutation is a hallmark of spontaneous mutagenesis resulting from the deamination of methylated cytidines (Skandalis *et al.*, 1994).

There were four (all +1) and eight (three +1 and five -1) frameshifts recovered from the pre-and post-etoposide spectra respectively. The majority of them (10 out of 12) included AT base pair and half of them occurred at sequences containing consecutive bases implicating misalignment and slippage mechanisms in their formation.

One complex mutation in post-etoposide spectra involved a replacement of a "T" at position 23 in exon 1 to an "AC" sequence which led to the formation of a direct 8 bp repeat with an upstream sequence (Table 2.7). Two tandem mutations in post-etoposide spectrum included a mutant (16E3-16) with two 62 bp and 26 bp deletions in exons 6 and 8 respectively. The other tandem mutation (mutant 24E2-9) had "CC" replaced to "TT" at positions 463-464 in exon 6 (Table 2.7). This mutation which may occur via oxidative damage (Reid *et al.*, 1993) or repair of cyclobutane pyrimidine dimers after UV light exposure (McGregor *et al.*, 1991) have been observed previously in an unexposed control spectrum recovered from a sample of a 33 year old male smoker (Curry *et al.*, submitted).

5. Discussion.

Chemotherapy and *hprt* MF. There have been several studies on the effect of chemotherapy on *hprt* MF (Table 2.4). Although each study commented on the extensive inter-individual variability, a significant increase in mean *hprt* MF in groups of patients has been observed after treatment in all studies (Branda *et al.*, 1991, Tates *et al.*, 1994, Hirota *et al.*, 1993, Ammenheuser *et al.*, 1988, Caggana *et al.*, 1991, Dempsey *et al.*, 1985). Many factors including the type of cancer, the drug and the dose were found to influence MF. For example, the alkylating agent cyclophosphamide was found to be associated with an increase in *hprt* MF in all studies, while etoposide and cisplatin chemotherapy was associated with a significant decrease in MF in 3 patients in one study (Tates *et al.*, 1994).

There was no increase in mean *hprt* MF in 12 SCLC patients receiving etoposide chemotherapy in our study (Table 2.3), although 33% of patients had higher MF during

the treatment (Figure 2.1). Our results are somewhat similar to another study of a group of germ cell tumor patients who received chemotherapy with either cisplatin or carboplatin in combination with etoposide or VAB-6 where the induction of *hprt* mutation was only marginal (Perera *et al.*, 1992). Interestingly, cisplatin and VAB-6 regimen that contained cyclophosphamide appeared to have a greater effect on MF than platinum plus etoposide treatment in that study.

The lack of an increase in the *hprt* MF after etoposide chemotherapy in our study may be attributed to several factors. For examples, the patients were elderly (the oldest age group studied (Table 2.4)). The relatively high MF of the pre-treatment samples may mask the possible mutagenic effect. It is possible that the same treatment regimen would give a positive result when studied in younger patients. In addition, the high MF may in part be attributed to the low CE (CE in half of the pre-treatment samples were <10%) (Figure 2.1) in our subjects. The low CE could be a cumulative result of old age, long smoking history, and possibly disease, and could have confounded the data.

Based on the analysis of data obtained from split and repeat blood samples it has been shown that individual *hprt* MF can vary considerably (2-8-fold) over time with repeat samples having the most variation (Cole *et al.*, 1994). This reflects the complex nature of T-cells, such as T-cell turnover, differences in T-cell subsets and their clonal potential (Cole *et al.*, 1994, Dubeau *et al.*, 1994). In our experiments with split samples there was up to a 6-fold fluctuation in MF and a 7.5-fold fluctuation in CE when cryopreserved samples were used in both cases and up to an 11-fold fluctuation in MF and a 8-fold fluctuation in CE when fresh and cryopreserved cells were compared (data not shown). To minimize the effect of cryopreservation, media, and handling conditions, samples

from individual patients were processed simultaneously when it was possible. However, in our samples the 95% confidence intervals for MF, which are calculated based on CE and the number of cells plated (Branda *et al.*, 1993 (1)), are often very large and there were no repeat samples from the individual patients where the confidence intervals would not overlap (Figure 2.1). This makes it difficult to assess the fluctuation in MF in our patients over time.

Clonality could be an issue here. O'Neill *et al.* (1994) have determined that clonality does not appear to be a confounding factor in estimating *hprt* MF when the calculated MF are below $40x10^{-6}$. In about half of our samples the MF is above $40x10^{-6}$ (Figure 2.1). The enhanced MF reflects in part the low CE as in almost all of these cases the CE is <10% (Figure 2.1). However, the analysis of mutants revealed only one sample (22E-4) with 8 out of 14 sequenced mutants having an identical mutation. Five additional cases had two identical mutations in each sample with a range of 12-34 sequenced mutants (Tables 2.6 and 2.7). Thus, clonality does not appear to be a major confounding factor in this study.

The calculated MF values were not adjusted for clonality in our samples (Morley 1996).

Finally, although patients were followed up during treatment for a period from 0.7 to 5.3 months after the beginning of chemotherapy and 5 out of 12 patients were followed after receiving four or more etoposide cycles, more samples at an earlier or later time after the treatment may have been more informative. It is possible that mutations are expressed much later after treatment, though there have been controversial reports on the appearance and persistence of mutation (Table 2.4). The mean lymphocyte lifespan varies from several days in short-lived to 1.1 and 6.3 years in long -lived cells (Tates *et al.*,

1994, Bogen, 1993). This wide range allows for the detection of mutants within our sampling time assuming that they are induced and that the induced cells are present in the circulation.

It is, however, possible that etoposide chemotherapy does not effectively induce mutation that can be detected by the *hprt* T-cell clonal assay.

The cytotoxicity of etoposide is cell cycle phase-specific with quiescent cells being much less sensitive (Ferguson *et al.*, 1994, Cole *et al.*, 1994). Thus, 3 μM of etoposide has been shown to be highly toxic in human lymphocytes when administered at the S-phase of the cell cycle and the few cells that reached metaphase exhibited multiple chromosome rearrangements. However, the cytotoxicity and cell cycle arrest were greatly reduced when 85 μM etoposide was administered at the G_1-phase (Maraschin *et al.*, 1990). As DNA replication is essential for mutations to occur and mutations are fixed in dividing cells, it is possible that the lack of mutation induction after etoposide chemotherapy in patients' lymphocytes observed in our study is due to the fact that the majority of circulating T-cells (up to 99%) are in the G_0 phase of the cell cycle.

Patients were treated with monthly cycles of oral etoposide 50 mg twice a day for 14 days. Pharmacokinetic studies showed that this regimen produces etoposide peak plasma levels of 1 to 3 μg/ml, which remains throughout the treatment period (Greco 1992). *In vitro* studies have shown that etoposide can be mutagenic and clastogenic in several systems at the concentrations achievable *in vivo*. Thus, treatment of CHO cells for 16 h at a concentration 0.8-1.25 μg/ml has been shown to reduce survival from 68.1% to 27.5% and increase *hprt* MF up to 45-fold (Singh *et al.*, 1983). Up to 5-fold increase in mutant

frequency and gross chromosome alterations have been detected after exposure of L5178Y/tk$^{+/-}$ cells to etoposide at concentrations below 0.1 μg/ml (Ashby *et al.*, 1994). A significant increase in SCE frequency has been revealed in PHA stimulated human lymphocytes exposed even to 0.025 μg/ml of etoposide for 72 h (Tominaga *et al.*, 1986). The results of these *in vitro* and our *in vivo* studies suggest that primary cells may respond differently than immortalized cell lines to the mutation induction by etoposide. One difference may be that immortalized cells are less susceptible to apoptosis.

Etoposide and apoptosis. Our results after etoposide chemotherapy are similar to previous findings with platinum plus etoposide chemotherapy in which a significant decrease or no change in the *hprt* MF was observed in patients after treatment (Tates *et al.*, 1994, Perera *et al.*, 1992). The reason for the lack of mutation induction is unclear but may be due to the phenomenon of apoptosis. Etoposide has been shown to induce apoptosis in different cell systems *in vitro* including thymocytes (Walker *et al.*, 1991), fibroblasts (Mizumoto *et al.*, 1994), leukemic cell lines (Dubrez *et al.*, 1995), and T- and B-cells (Pette *et al.*, 1995, Fournel *et al.*, 1995). Apoptosis has also been detected in leukemic patients 8 to 24 h after the administration of chemotherapy including etoposide in one study (Li *et al.*, 1994), but in another study of children with leukemia apoptosis was not detected after treatment with etoposide and prednisolone (Matsubara *et al.*, 1994). Apoptosis would readily explain the absence of changes in MF in our study. Since circulating lymphocytes with etoposide induced DNA damage (and mutation) are good candidates for apoptosis. Due to their rapid removal, they would be undetected by the *hprt* assay.

Mutational spectra and etoposide. There have been only a limited number of studies where the molecular analysis of *hprt* mutants obtained from exposed individuals has been reported. Southern blot analysis showed a dose-dependent increase in the proportion of gross structural alterations in the *hprt* gene in radioimmunoglobulin therapy (RIT) treated cancer patients compared to untreated patients (40% *vs.* 20% and around 15% in normal non-exposed adult donors) (Nicklas *et al.*, 1991). Hirota *et al.* (1993) found the same increase in children with ALL treated with radiotherapy and multiple drug chemotherapy (23.1% *vs.* none in untreated healthy children). In both studies the *hprt* MF was significantly increased in treated patients. As a Topo II inhibitor and an inducer of DNA strand breaks, etoposide is anticipated to produce predominately gross deletions and rearrangements. More than 50 potential Topo II cleavage sites (Edwards *et al.*, 1990) can be mapped on the *hprt* gene including one within the exon 3 of the coding region. However, no increase in gross deletions or rearrangements was seen in mutants from treated patients analyzed by RT-PCR in our study. This is consistent with the lack of an increase in MF after treatment in our study.

The *hprt* spectrum of unexposed human population contains more than five hundred mutations, however, the "background" spectrum is by no means saturated. Novel mutations are being recovered as more mutants from unexposed subjects are being sequenced (Cole et al., 1994, Curry *et al.*, 1996). A considerable overlap exists between the "background" mutation spectrum and mutations recovered *in vivo* and *in vitro* after mutagenic exposure (Cole et al., 1994). These factors do not allow us to make definite conclusions on the spectra comparisons and possible effects of etoposide exposure. However in our study, some trends can be pointed out which may suggest a difference

between the "background" spectrum collected in the *hprt* database and the spectra of mutations from a group of SCLC patients receiving etoposide chemotherapy.

The overall spectrum of 147 mutations recovered from etoposide- treated patients was found to be similar to the spectrum of 56 mutations from the pre-treatment samples of these patients and to the spectrum of 337 mutations from population with no known genotoxic exposure (Table 2.8). Statistically significant differences were seen in the distributions of exon losses (Table 2.9). Because the underlying mutations were not identified in most of these mutants the clonality of mutants with the same exon losses recovered from the same sample is not excluded and this might have affected the results. However, a clear trend was seen in the reduction of mutations affecting exon 4 and an increase in mutations affecting exon 1 in post-etoposide spectrum compared to unexposed control spectrum. Exon 4 is one of the two most affected exons in the *hprt* gene among the splice mutants in the control spectrum with reported 29% (Cole et al., 1994) and 14% (Curry *et al.*, submitted) of splice mutants having alternative splicing of this exon. Only one out of 129 mutants in the unexposed control spectrum had a mutation (36 bp deletion) leading to the alternative splicing of exon 1 (Curry *et al.*, submitted). In our post-etoposide spectrum six mutants, or 9% of splice mutants, had alternative splicing of exon 1 (Table 2.9). All six mutations were different. Two mutants (15E2-2 and 15E2-8) came from the same sample and the 16 bp deletion in the first mutant was enclosed by the 37 bp deletion in the second mutant (Table 2.7). Interestingly, four of the six mutants had deletions of various sizes affecting the splice sites of exon 1, two of which had no flanking, repeat sequences. Such deletions affecting the correct splicing of exon 1 were rarely recovered in the control unexposed spectrum (one out of 33 deletion mutants)

which was another noticed difference between this and the post-etoposide spectra (Table 2.10).

Two post-etoposide mutants (17E2-25 and 16E3-4) had deletions clustered at the 3' end of exon 3. The clustering of deletion mutants in this region have been reported in people accidentally exposed to ionizing radiation (Skandalis *et al.*, 1997), cancer patients with radioimmunoglobulin therapy (Skandalis *et al.*, 1995) and after in *vitro* malathion exposure (Pluth *et al.*, 1996). Possible hairpin-loop structure forming in this region (Pluth *et al.*, 1996) and a potential Topo II binding site may be linked to the clustering of the deletions. However, the fact that our exon 3 deletions map outside of the proposed hairpin-loop and the potential Topo II site and because deletions in the same region have been recovered in the unexposed control spectra (Curry *et al.*, submitted) suggests that these deletions were unlikely induced by etoposide exposure (Tables 2.7 and 2.10).

Several etoposide mutants induced *in vitro* have been studied at the molecular level. Berger *et al.* (1991) analyzed 10 induced *hprt* mutants from V79 cells and demonstrated by Southern blotting that most of them were partial deletions and/or rearrangements of the *hprt* gene. In L5178Y/tk$^{+/-}$ cells etoposide induced mostly small colony mutants and primarily large scale losses of at least 6.3 kb of the functional *tk* gene as shown by the Southern blot analysis of 96 mutants (Ashby *et al.*, 1994). Out of 65 teniposide-induced mutations at the hemizygous *aprt* locus of CHO cells 63% were deletions, duplications and insertions of various sizes, with the majority being less than 20 bp (Han *et al.*, 1993). Moreover, a significant correlation has been found between the breakpoints of small deletions and duplications and sites of teniposide-stimulated Topo II cleavage sites *in vitro*. The discrepancy seen between these and our studies of etoposide mutagenesis

probably reflects the difference in cell types, gene targets as well as *in vitro vs. in vivo* situations.

Recently, etoposide has been shown to cause illegitimate VDJ recombination in a dose-dependent manner in human lymphoid, but not myeloid leukemic cells measured by site-specific deletion of exon 2 and 3 in the *hprt* gene (Chen *et al.*, 1996). Interestingly, the frequency of the same exon 2 and 3 deletion mutation was found elevated in germ cell tumor patient treated with *cis*-platinum and etoposide compared to the frequency of that mutation in healthy untreated age-matched donors, although the number of mutants examined (22) from the patient was relatively small (Fuscoe *et al.*, 1992 (1)). In our mutant collection the proportion of exon 2 and 3 losses from the cDNA increased but not significantly in post-treatment compared to pre-treatment mutants or control spectra (Curry *et al.*, submitted) (12 out of 147 total mutations (8.2%) *vs.* 3 out of 56 total mutations (5.3%) or *vs.* 18 out of 337 total mutations (5.3%)) (Tables 2.6 and 2.7). It should also be noted that not all of these mutants might have VDJ recombinase site-specific genomic deletion of exons 2 and 3 as other mutations in splice sites could have contributed to the loss of these exons in our mutants with uncharacterized splice mutation.

Significant enhancement of AT >TA transversions accompanied by a decrease in the number of GC>TA transversions was noticed in post-etoposide spectrum (Table 2.11). The reason for these changes is unknown. Transversions at AT base pairs were found to be a predominant type of single base substitutions in the *hprt* gene after exposure to alkylating agent N-ethyl-N-nitrosourea (ENU) (Jansen *et al.*, 1995) and in the p53 gene in some cancers (Hartmann *et al.*, 1995). GC>TA transversion is the predominant type of

mutation found in p53 and K-ras genes in human lung cancer consistent with the mutational spectra of benzo[a]pyrene found in tobacco smoke (Johnson *et al.*, 1993, Cherpillod *et al.*, 1995). No increase in GC>TA transversions was found in our mutant collection from SCLC patients most of whom are heavy smokers. This observation is in agreement with previous findings that smoking does not affect the *hprt* mutational spectra (Vrieling *et al.*, 1992). An increase in the proportion of AT>TA transversions in post-etoposide mutational spectrum may thus indicate the direct or indirect mutagenic effect of etoposide, however, no mechanistic explanation can be offered at this moment.

6. Conclusion.

This study found no increase in mutation after etoposide chemotherapy in SCLC patients (age mean 74.8 ± 6.0; range 66-83) using the *hprt* T-cell cloning assay. The lack of an increase in mutant frequency, however, does not mean that the cytotoxic treatment did not result in genotoxic damage in these patients. The non-random clastogenic effect of etoposide *in vitro* in human lymphocytes has been demonstrated in previous studies with chromosomes 1, 11 and 17 overaffected (Maraschin *et al.*, 1990). Etoposide, together with other Topo II inhibitors, has been linked to sAML with specific rearrangements of the chromosomal band 11q23. Therefore, we conclude that a study of chromosomal aberrations in patients after etoposide chemotherapy may provide better insight into the adverse genotoxic effects of etoposide than does the measurement of the *hprt* mutant frequency in T-lymphocytes. The *hprt* T-cell cloning assay, though useful in some studies, may not be informative in the assessment of the genotoxic effect of

etoposide treatment due to the complex nature of T-cells and their yet unknown response to etoposide chemotherapy.

CHAPTER III. *IN VIVO* CLONAL EXPANSION OF *hprt* MUTANTS AND VARIOUS TCR-γ GENE REARRANGEMENTS IN THE *HPRT* MUTANT T-CELL CLONES DETECTED IN SCLC PATIENTS UNDERGOING ETOPOSIDE CHEMOTHERAPY

1. Abstract.

One complication of the *hprt* T-cell clonal assay is an *in vivo* clonal expansion that can contribute to the overestimation of individual MF and bias the mutational spectra. Clonality of the *hprt* mutants selected from the SCLC patients undergoing etoposide chemotherapy was assessed using the restriction fragment length polymorphism (RFLP) and a new sequence analysis of a PCR amplified T-cell receptor γ (TCR-γ) gene . While in most samples clonality did not seem to have a significant effect on the *hprt* MF, in one patient (3E) a large mutant clone with most mutants having a +C frameshift after position 329 in exon 4 was detected. This expanded clone was found to persist for at least 4 months at the time of etoposide exposure and, possibly, accumulated additional mutations during this expansion. After correction for clonality the MFs in this patient were reduced substantially and fell into the normal range of other SCLC patients ($958x10^{-6}$ *vs.* $63x10^{-6}$ before treatment and $151x10^{-6}$ *vs.* $47x10^{-6}$ after treatment) . In another patient (22E), 8 out of 14 sequenced mutants were found to bear the same 11bp deletion in exon 2 flanked by 6 bp repeats. The TCR analysis of these mutants showed that a rarely used variable gene V9 was utilized in the TCRγ-gene rearrangement. Clonality was also assessed in mutants with identical splice errors selected either from the same or repeat blood samples from patients 7E and 1E. It was found that such mutants are unlikely to be clonal which is

consistent with the fact that various mutations can lead to the same splice errors or exon skipping events. The new method for the detection of mutant clonality based on the sequencing of the PCR- amplified TCRγ-gene rearrangements proved to be valuable and more informative than the RFLP method. Using the sequencing method it was found that in one mutant a pseudo-gene V7 was used in the TCR-γ gene rearrangement. In another mutant a mixture of two variable genes V2 and V8 was found in the rearrangement indicating that two alleles were rearranged differently. Additionally, two mutants selected from two different patients were found to have identical rearrangements. Significance of these findings is discussed.

2. Introduction.

Mutation at the *hprt* locus occurring *in vivo* in humans can be determined by a T-cell clonal assay (Albertini *et al.*, 1982). This assay quantifies the frequency of 6TG-resistant mutants, which is a measure of the *in vivo hprt* mutation frequency. One source of variation in the estimation of the *hprt* MF in individual subjects or in populations is the complex dynamics of T-cell populations. In adults, the total number of T-cells can be regarded as remaining relatively constant, although this apparent constancy conceals underlying dynamic expansion and contraction of lymphocyte subpopulations. The gain of lymphocytes due to proliferation occurring in response to antigenic stimulation is matched by an equal loss owing to death, perhaps due to apoptosis. As a result, the individual lymphocytes present at any particular time will be represented at a later time point by either no descendants, by single descendants or by clones of various sizes dependent on the extent of expansion and contraction undergone by the clones. This is

true for any T-cell subpopulations, including the *hprt* mutant cells. When a large *hprt* mutant clone is present in a particular individual at some time point, a highly elevated *hprt* MF would be detected in this individual even though the mutation rate may not be elevated. Furthermore, if the *hprt* mutation occurred prior to the clonal proliferation, the mutational spectra will have a hot spot produced by the identical mutation recovered multiple times in clonal *hprt* mutants. These events not only contribute to the variation in the *hprt* MF determination but can also be misinterpreted as an effect of mutagenic exposure. The understanding of proliferation dynamics of *hprt* mutants and their impact on the *hprt* MF and mutational spectra are therefore important in studies monitoring human genotoxic exposures.

The clonality of *hprt* mutants can be determined by the analysis of their TCR gene rearrangements. The specificity of T-cells is determined by these rearrangements which occur during T-cell maturation in the thymus by the action of VDJ recombinase. T-cells remain in a G_0 phase *in vivo* until they are activated by specific antigens, enter the cell cycle and proliferate. Any T-cell with a particular set of rearranged TCR genes responds to an activating signal and proliferates forming a clonally expanded population. All subsequent daughter cells will have the same TCR genes rearrangements and specificity. If the original mature cell sustains an *hprt* mutation, a clone of *hprt* mutant cells will arise where all cells will have the same TCR gene rearrangements. By analyzing these rearrangements one can determine the clonality of *hprt* mutants. In an individual, two or more *hprt* mutants showing the same TCR gene rearrangements represent *in vivo* clonal proliferation. In general, each unique TCR gene rearrangement pattern in the *hprt* mutant defines an independent *in vivo hprt* mutational event. The possibilities also exist for

special cases with the repetitive occurrences of independent *in vivo hprt* mutations in the same TCR gene defined T-cell clone.

Analysis of TCR gene rearrangements has shown that *in vivo* clonality is a common occurrence in the 6TG-resistant T-cell population (O'Neill *et al.*, 1994). The proportion of mutants with different TCR gene patterns in individual samples can range from 100% to 9% (0-91% clonality). However, the distribution of clonality among *hprt* mutants is such that 88.2% of the samples show 30% clonality or less (70%-100% different TCR patterns). Overall 90% of mutant clones analyzed were the result of independent mutations and 10% were siblings (Cole et al., 1994). Moreover, when the estimated *hprt* MF was less than $40x10^{-6}$, the MF corrected for clonality was always within one standard deviation of the measured MF indicating that clonality does not have a significant effect on MF in cases when the MF is less than $40x10^{-6}$.

Analysis of TCR gene rearrangements has led to the suggestion that in adults, most *hprt* mutations are occurring in post-thymic cells. In the majority of cases clonal mutants had the same *hprt* mutations indicating that the mutation occurred in the post-thymic cell when the rearrangement of TCR genes had been completed. Only in rare cases are identical *hprt* mutations recovered in clonally independent mutants suggesting that the *hprt* mutation occurred in the pre-thymic T-cell, possibly a germ cell (O'Neill *et al.*, 1994). The fact that all analyzed wild type T-cells show unique TCR gene rearrangement pattern (0% clonality) and that only *hprt* mutants may represent clonally expanded clones (64.5 % clonality) indicates that *hprt* mutations may occur preferentially in cells that are dividing in response to antigenic stimulation (O'Neill *et al.*, 1994). Further support for the preferential occurrence of *hprt* mutations in post-thymic proliferating cells is provided

by the finding of both wild type and *hprt* mutant cells with the same TCR gene rearrangement pattern (O'Neill *et al.*, 1994).

Analysis of TCR gene rearrangements in *hprt* mutants recovered from repeat blood samples from individuals has been used to estimate the life span of *in vivo* arising *hprt* mutants. A relatively short life span (1-3 months) was estimated in one study, while in other cases mutant clones persisted for 10 months-2 years or more (Cole *et al.*, 1994). There is a controversial evidence for the selection against $HPRT^-$ lymphocytes *in vivo*. The main argument supporting the negative selection is the demonstration that, while most tissues in Lesch-Nyhan heterozygote females have 50% HPRT+/HPRT- cells, the proportion of *hprt* mutant lymphocytes is considerably less than 50% (5%-10%) (Cole *et al.*, 1994). Contrary, other studies demonstrated up to 40% of *hprt* mutant lymphocytes in Lesch-Nyhan heterozygote females and, in addition, all blood-derived cells in Lesch-Nyhan heterozygotes were found to be under-represented for *hprt* mutants. This suggests that selective disadvantage is occurring at an early stage in hematopoietic development and affects all blood lineages. Bone marrow and brain are the only organs that are particularly affected by the HPRT deficiency since almost no *de novo* nucleotide synthesis occurs there. Because bone marrow cells require HPRT activity for normal function, especially in a rapidly proliferating stage, *hprt* mutants could well be at a selective disadvantage during early foetal development. This reason can explain the low proportion of *hprt* mutants in all blood cells of heterozygotes and the low MF found in cord blood, but it does not imply a selective disadvantage for HPRT- post-thymic lymphocytes in older subjects. It was suggested that the persistence of *hprt* mutant clones

is a function of the nature of the particular T-cell subset and position in the lineage of the cell in which mutation occurred (Cole et al., 1994).

The most interesting cases of clonal proliferation are those with exceptionally large mutant clones. In one such case the *hprt* MF was seen to rise over a four-year period in an individual from 80 to> $600x10^{-6}$ (Nicklas *et al.*, 1988). Analysis of the TCR rearrangements showed that over 90% of the mutants were descendants of the same mature T-cell precursor (Nicklas *et al.*, 1988). These mutants clearly were not at a selective disadvantage. When the observed MF was adjusted for clonality a near "normal" frequency of mutants ($13-44x10^{-6}$) was found.

One of the methods designed to determine the clonality of the *hprt* mutants is based on the RFLP analysis of PCR amplified rearranged TCR -γ gene (de Boer *et al.*, 1993). The PCR amplified junction between rearranged variable and joining gene segments is subjected to restriction digest by two enzymes BstN1 and RsaI and the digestion pattern is revealed by separating the bands on the agarose gel. The same pattern obtained after digestion with two restriction enzymes confers the clonal relationship of the mutants. The different patterns indicate that the mutants are clonally independent.

In this study a new method was introduced which used automated sequencing rather than RFLP in the analysis of the PCR-amplified TCRγ-gene segment. Using both methods, clonality of groups of mutants with identical *hprt* mutations was assessed in SCLC patients undergoing etoposide chemotherapy. In one patient, the large mutant clone was found which greatly elevated the *hprt* MF. The clone was found to persist for at least 4 months and possibly had accumulated additional *hprt* mutations during the *in*

vivo expansion. In another patient, a rarely used variable gene V9 was found to be involved in the TCRγ-gene rearrangement. It was also determined that mutants with identical splice errors such as whole or partial exon losses are likely to be clonally independent. The usefulness and superiority of the sequencing method was demonstrated.

3. Materials and methods.

Sample collection, T-cell clonal assay and mutation identification. The patient sample collection, T-cell clonal assay and the sequence analysis of RT-PCR amplified *hprt* cDNA were described in Chapter II.

PCR amplification and RFLP analysis of the TCRγ-gene. Pellets of 2,000 cells were resuspended in 10 μl of water, vortexed and spun down. Then PCR buffer (de Boer *et al.*, 1993), 20 pmol of each primer V1 and J1 (de Boer *et al.*, 1993), 0.5 ul of 25 mM dNTP mix, 2.5 U *Taq* polymerase and water were added to the cells to a total volume of 50 ul. PCR reactions performed using the following protocol: 5 min at 95°C followed by 5 cycles of 2 min at 95°C, 2 min at 60°C, 5 min at 72°C (short program) and 25 cycles of 20 sec at 94°C, 30 sec at 55° C, and 1 min at 72°C, followed by 10 min at 72°C (long program). 2 ul of this reaction was used in the second PCR reaction with inner primers V2 and J2 (de Boer *et al.*, 1993) in a 100 ul reaction volume using the following program: 4 min at 94°C, 30 cycles of 5 sec at 94°C, 5 sec at 55°C, 1 min at 72°C followed by 5 min at 72°C. DNA was isopropanol precipitated and resuspended in 36 ul of water. Restriction digestion was performed with BstN1 and RsaI (New England Biolabs) overnight according to manufacturer's protocol. The restriction fragments were separated

on a 5% NuSieve (FMC Bioproducts) agarose gel. Mutants were judged as clonally independent if they had different patterns of fragments after digests with either of the two restriction enzymes. Alternatively, mutants were considered clonally related if the pattern of fragments was the same with both enzymes.

In the case of mutants from sample 22E-4 genomic DNA was extracted from the cell pellets and 250 ng of genomic DNA was used in a single PCR reaction (second PCR reaction described above) using primer V9 (5'-CAG CCC GCC TGG AAT) and J1 . The genomic DNA was extracted using the following protocol: approximately $0.2x10^6$ cells were placed in a Wheaton 15ml douncer (Baxter) containing 5 ml of ice-cold cell lysis solution (CLS) (10ml TrisHCl, pH 8.3, 140 mM NaCl, 0.35 M sucrose, 1mM EDTA, 1% Triton X-100) and dounced up to 10 times, first with the loose pestle then with the tight pestle, yielding a crude nuclei preparation; this mixture was filtered through a 100-500 micron nylon mesh filter into a sterile 50ml conical tube and centrifuged for 12 min at 1100xg at 4°C; the supernatant was decanted and 75 µl of phosphate buffered saline with EDTA (12.3 mM Na_2HPO_4 , 1.4 mM KH_2PO_4, 13.7 mM NaCl, 2.7 mM KCl, 10 mM EDTA, pH 8.0) was added to the pellet; the equal volume of proteinase K/SDS solution (2mg/ml proteinase K, 2% SDS, 100 mM EDTA pre-warmed to 50°C for 5 min) was added to the pellet, mixed gently and incubated for 15 min at 50°C in the conical 50 ml centrifuge tube; the solution was carefully transferred with a wide-bore pipette tip onto a 0.45 µm pore size type HA filter (Millipore) floating over 35 ml of TE solution in a petri dish; the samples were dialyzed for 48 hours at room temperature. The DNA then was transferred with a wide-bore pipette tip to a microcentrifuge tube. The concentration of

the template was measured using a TKO 100 Fluorometer (Hoefer Scientific Instruments, San Francisco) according to the manufacture's protocol.

Sequencing of the TCRγ-gene and the antomation of the sequencing method. The sequencing was performed using ALF automated DNA sequencer (Pharmacia) using ReadyMix Gel-ALF Grade (Pharmacia) and following reaction protocols described in the AutoCycle Sequencing kits (Pharmacia). 2.5 pmol of 5'-fluorescein labeled V1 or V9 primer was added to a 100ng of template in a total volume of 6 μl. A reaction mixture containing a total volume of 20μl of water, buffer (300mM Tris pH 9.0, 300mM KCl, 50mM $MgCl_2$) and Taq polymerase was added to the template/primer mix. Then 6 μl of this total mixture was added to 4 μl of each of the four stop mixes (60μM of each dNTP and either 0.8 mM of each ddATP or ddTTP or 0.4mM of ddCTP or 0.08mM of ddGTP in the A, T, C and G stop mixes respectively). The following PCR reaction was used: 2 min at 94°C followed by 25 cycles of 20 sec at 94°C, 10 sec at 50°C and 1min at 72°C. After adding 5 μl of the formamide/dextran blue solution, the sequencing reactions were denatured at 94°C for 2 min and quickly placed on ice. Ten μl of the reaction was loaded on the sequencing gel.

In an automated sequencing method the sequencing reactions were performed only with an A stop mix so that only adenine sequence was obtained for each TCRγ-gene. Denatured adenine sequencing reactions from four mutants were loaded onto A,C,G and T lanes of the sequencing gel. Each of the four colours (green for A, red for C, yellow for G and red for T in the normal sequencing reaction) represented the position of adenines in the corresponding TCRγ-gene sequence of each of four mutants. When the colours did

not match, this indicated the difference in sequence and was judged as an alternative TCRγ-gene rearrangement in the mutants.

4. Results.

Patient 3E. *hprt* MF, clonality and mutation frequency. Four blood samples were collected from patient 3E (male, 77 years old, 55 years smoking history). One pre-treatment sample (3E-0), a sample after two etoposide cycles (3E-2), one extra sample taken 4 days after the completion of the third cycle (3E-3+), and regular samples after the third (3E-3) and the fourth etoposide cycles (3E-4) (Table 3.1). Sample 3E-4 was split and approximately half of the recovered cells were pre-incubated immediately while the remaining cells were cryopreserved. At a later date these cryopreserved cells and the cells from the remaining samples from this patient were thawed and pre-incubated simultaneously (Table 3.1). The MF results from this patient samples had several unusual characteristics (Table 3.1). First, the MF in sample 3E-4 cultured with fresh cells was unusually high ($151x10^{-6}$). Second, when cryopreserved cells were used in the experiment, the MFs were even higher (ranged from $546x10^{-6}$ to $958x10^{-6}$) in all samples except for sample 3E-2, where the MF was in the normal range ($25x10^{-6}$). Third, there was a great difference in MF in sample 3E-4 when either fresh ($151x10^{-6}$) or frozen ($765x10^{-6}$) cells were used (Table 3.1).

Sample 3E-4. It appeared likely that *in vivo* clonal proliferation of *hprt* mutant T-cell(s) took place, which resulted in the highly elevated MF values. To test this, clonality of mutants was investigated. Clonality studies allowed for the correction of MF that became the frequency of independent mutations.

Table 3.1 The results of the *hprt* T-cell cloning assay in pre-treatment (3 E-0 and 7 E-0) and post-treatment samples from SCLC patients 3E and 7E.

Sample ID	Sample date	non-selective wells		selective wells		CE	MF ($x10^{-6}$)	95% confid. interv. ($x10^{-6}$)	
		+	total	+	total			upper	lower
3 E-0	18.06.93	84	192	148	176	0.19	958	1274	721
3 E-0[c]		84	192	20	176	0.19	63	103	39
3 E-2[a]	18.08.93	82	192	12	264	0.19	25	46	14
3 E-3[b]	09.01.93	77	192	230	336	0.17	675	879	519
3 E-3	15.09.93	69	192	271	488	0.15	546	713	418
3 E-4 (1)	13.10.93	68	192	43	64	0.15	765	1136	515
3 E-4 (2)[c]	13.10.93	115	232	128	440	0.23	151	194	117
3 E-4 (2)[c]		115	232	45	440	0.23	47	67	33
7 E-0	23.06.93	44	192	8	140	0.09	68	144	32
7 E-1	28.07.93	32	192	3	140	0.06	36	116	11
7 E-2[a]	18.08.93	64	192	27	144	0.14	154	241	98
7 E-3	09.07.93	21	192	20	536	0.04	98	182	53
7E-4[d]	16.09.93	41	192	14	232	0.08	78	143	42
7 E-4 (1)	20.10.93	36	192	5	160	0.07	46	117	18
7 E-4 (2)[c]	20.10.93	23	192	27	376	0.04	175	306	100
22 E-0[e]	05.10.95	76	192	13	248	0.17	32	58	18
22 E-4[e]	19.04.95	96	192	17	224	0.23	34	57	20
22 E-4[c]		96	192	10	224	0.23	20	38	10

[a] Samples 3E-2 and 7E-2 were collected and processed on the same day.
[b] This 3E-3 sample was taken 4 days after the third etoposide cycle.
[c] The MF for these samples was corrected for clonality.
[d] This 7E-4 sample was taken 2 days before the end of the fourth cycle.
[e] Experiments were done with fresh cells from these samples. Experiments were done with cryopreserved cells from all other samples.

Table 3.2 Sequenced *hprt* mutants from two SCLC patients 3E and 7E undergoing etoposide chemotherapy from a pre-treatment sample 3E-0 and post-treatment samples 3E-4 and 7E-2.

Mutant ID	Mutation	Position	Exon	AA change	cDNA alteration	Sequence	
3E4 (128)							
3E4-83	TS G>A	3	1	Met>Ile		tccgttATG GCG	
3E4-12	TS G>A	143	3	Arg>His		GAA CGT CTT	
3E4-18	TS G>A	197	3	Cys>Tyr		CTC TGT GTG	
3E4-80	TS G>A	212	3	Gly>Asp		GGG GGC TAT	
3E4-118	TS G>A	212	3	Gly>Asp		GGG GGC TAT	
3E4-77	TS G>A	212	3	Gly>Asp	exon 2-3 loss in some	GGG GGC TAT	
3E4-85	TV T>G	+2	I5		IS 67 bp of I5, exon 2-6 loss in some	GAA gtaa..aaaggtaa	
3E4-29[a]	F +C	329'	4			CAG TCA ACA	
3E4-89	F +C	329'	4			CAG TCA ACA	
3E4-56	F +C	329'	4			CAG TCA ACA	
3E4-88	F +C	329'	4		exon 4 loss	CAG TCA ACA	
3E4-87	F +C	329'	4		exon 4 loss in some	CAG TCA ACA	
3E4-108	F +C	329'	4		exon 2-5 loss in some	CAG TCA ACA	
3E4-49	F +C	329'	4			CAG TCA ACA	
3E4-32[b]	F +C	329'	4			CAG TCA ACA	
3E4-93[c]	+C (?)	329'	4		exon 4 loss in some	CAG TCA ACA	
3E4-34[d]	+C (?)	329'	4		exon 4 loss in some	CAG TCA ACA	
3E4-111[e]	SU				exon 4 loss		
3E4-40	C TS T>C, TV T>G	618, 299	9, 3	no change, Ile>Ser		GTT TGT GTC,	TTT ATC AGA
3E4-69	NF						
3 E-0 (148)							
3E0-21	TS G>A	212	3	Gly>Asp		GGG GGC TAT	
3E0-76	F +C	329'	4			CAG TCA ACA	
3E0-22	F +C	329'	4			CAG TCA ACA	
3E0-77	F +C	329'	4			CAG TCA ACA	

3E0-19	F +C	329'	4			CAG TCA ACA
3E0-83	F +C	329'	4			CAG TCA ACA
3E0-75	F +C	329'	4			CAG TCA ACA
3E0-82	F +C	329'	4			CAG TCA ACA
3E0-120	F +C	329'	4		exon 2-3 loss in some	CAG TCA ACA
3E0-30	F +C	329'	4			CAG TCA ACA
3E0-38	F +C	329'	4			CAG TCA ACA
3E0-74	F +C	329'	4			CAG TCA ACA
3E0-121	F +C	329'	4			CAG TCA ACA
3E0-95	SU				exon 4 loss	
3E0-56	SU				exon 4-8 loss	
7E2 (24)						
7E2-28	TS G>A	212	3	Gly>Asp		GGG GGC TAT
7E2-32	F +C	329	4			CAG TCA ACA
7E2-15	F +C	329	4			CAG TCA ACA
7E2-29	TV A>T	496	7	Lys>stop		GTG AAA AGG
7E2-12	SU				exon 8 loss	

Mutants [a], [b], [c], [d], and [e] had other mutations or cDNA alterations in a proportion of cDNA molecules or cells, which are described in Table 3.3.

Twenty of the 128 selected mutants from sample 3E-4 (fresh cells) were sequenced. Eight out of 20 sequenced mutants were found to have the same mutation, a +C frameshift following position 329 of exon 4 (Table 3.2). Support for the clonal relationship between +C mutants was provided by the analysis of TCRγ-gene rearrangement. The RFLP analysis of 3E-4 mutants showed that 6 out of 8 mutants with +C frameshift (two other mutants 3E4-89 and 3E4-56 were not analyzed) had the same band pattern after digestion with two restriction enzymes confirming their clonality (Figure 3.1). Comparison of the observed band patterns with known restriction digest patterns for various variable genes showed that V8 gene was used in the rearrangement in this expanded clone with a +C frameshift (Figure 3.1 and Figure 1.10).

To investigate whether there were any other clonal mutants in sample 3E-4, RFLP analysis was extended to other mutants from this sample. Although the band patterns on the RFLP gels are sometimes ambiguous, several other observations were made. The RFLP analysis on other mutants showed that four of them (mutant 3E4-69 where mutation has not been found and three other mutants (3E4-93, 3E4-34 and 3E4-111) which had exon 4 missing either completely or from the proportion of cDNA molecules) had the same restriction patterns as the mutants with +C frameshift suggesting that these four mutants also belong to the +C clone (Figure 3.1). While the loss of the fourth exon or the mixture of two cDNA populations prevented the identification of the +C mutations in the latter mutants, the absence of the +C mutation in mutant 3E4-69 is unclear. The other three mutants analyzed by RFLP (3E4-118, 3E4-77 and 3E4-83) had different band patterns consistent with different types of mutations found in them. Mutants 3E4-118 and

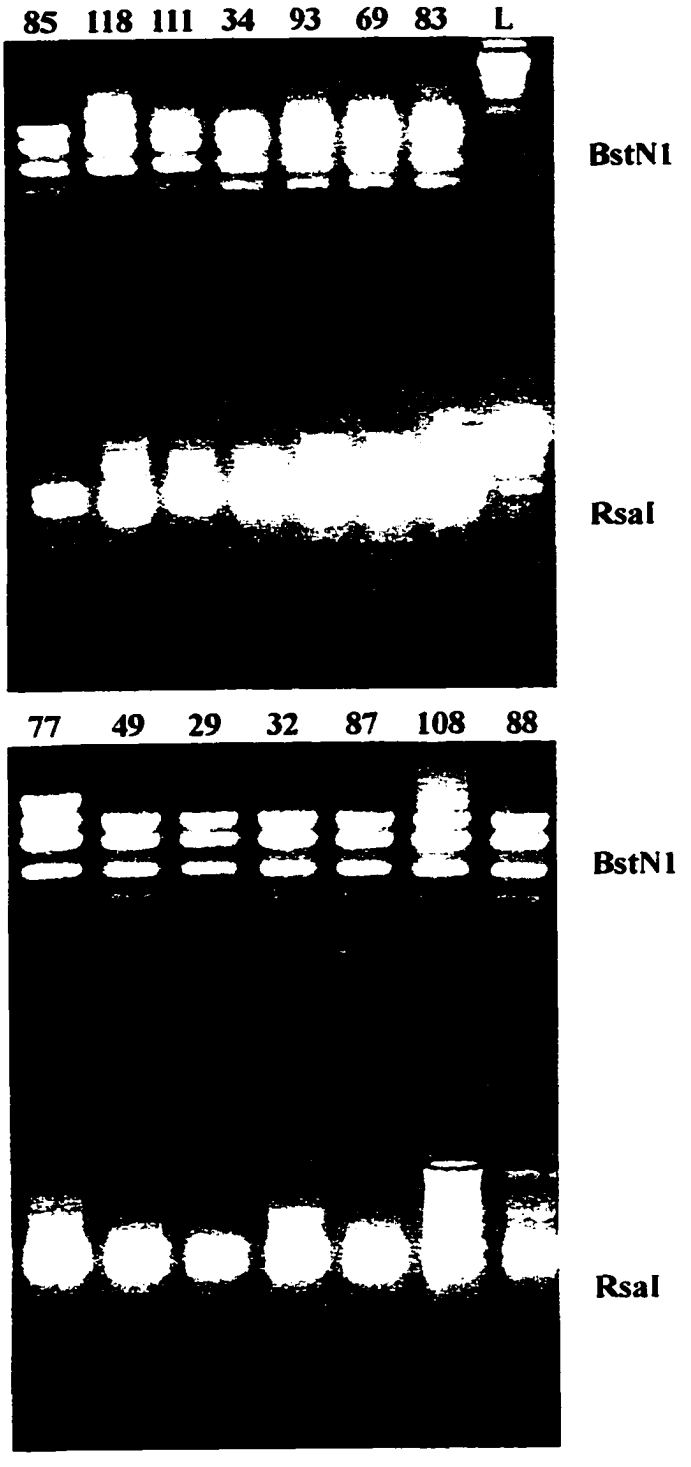


Figure 3.1 RFLP analysis of the TCR-γ gene rearrangements in *hprt* mutants from sample 3E-4 (mutant number is shown on the top of the gel). PCR-amplified TCR-γ gene was digested with two restriction enzymes BstN1 and RsaI and fragments were separated on 5% NuSieve agarose gel. L, 1kb DNA ladder (GibcoBRL).

3E4-77, both with GC>AT transitions at position 212, had similar or the same restriction patterns suggesting their clonality (Figure 3.1). The band pattern after RsaI digestion suggests the use of V4 gene in the rearrangement, however, the band pattern after BstN1 digestion, although identical in both mutants, has extra bands indicative of a mixture of V4 and V8 genes (Figure 3.1). Alternatively, the pseudo-gene might have been used in the rearrangement that has a distinct not yet determined digestion pattern. The last mutant analyzed, 3E4-85, appeared to have band patterns similar to that in the +C mutants, however, possible slight difference in the size of the smallest band after BstN1 digestion and the appearance of two extra intermediate bands on a RsaI pattern indicate the clonal independence between this mutant and +C mutants (Figure 3.1). No +C mutation was detected in mutant 3E4-85 and it was considered clonally independent for further analysis of mutation frequency (Table 3.2).

Based on the RFLP analysis, 12 out of 20 sequenced mutants from sample 3E-4 belong to one clone with a common +C frameshift mutation. Two other mutants belong to a separate clone with a GC>AT transition at position 212. Although the RFLP analysis was not done on mutant 3E4-80, this mutant has a GC>AT transition at position 212 and is probably related to the other two with the same mutation. All three mutants were considered clonal for the further analysis of mutation frequency. Therefore, only 7 out of 20 mutants were counted as independent mutations in sample 3E-4. Assuming the same distribution of independent mutations in the whole selected mutant population, the MF calculated previously for this sample (151×10^{-6}) can be adjusted for clonality and the frequency of independent mutations becomes 47×10^{-6}, which is within the range of MF found in post-treatment samples from other SCLC patients (Table 3.1, Chapter II).

Sample 3E-0. The MF in pre-treatment sample 3E-0 was also unusually high ($958x10^{-6}$) suggesting the presence of a large *hprt* mutant clone in this sample as well (Table 3.1). To test this, 15 of 148 selected mutants from sample 3E-0 were sequenced. Twelve of them were found to have a +C frameshift following position 329 in exon 4 (Table 3.2). The other two sequenced mutants (3E0-95 and 3E0-56) had exon 4 and exons 4-8 missing from their cDNA respectively and the last mutant had a GC>AT transition at position 212 (Table 3.2). Clonality of +C mutants in the pre-treatment sample was demonstrated by the sequencing method. All 5 analyzed +C mutants had identical sequence of their TCRγ-gene rearrangement confirming their clonality (Table 3.3). Comparison of the obtained sequence with the genomic sequence of the variable TCRγ-genes confirmed the use of the V8 gene in the rearrangements in these mutants.

Sequence analysis of the TCR-γ gene was also done on other mutants from sample 3E-0 with no apparent +C frameshift mutation. Mutant 3E0-56 had the same TCR-γ gene sequence as did mutants from the +C clone suggesting their clonal relationship (Table 3.3). This mutant had exons 4-8 missing from its cDNA, which prevented the identification of the +C frameshift mutation in exon 4 (Tables 3.2 and 3.3). Although the sequence analysis of the TCRγ-gene rearrangement was not done on mutant 3E0-95 with exon 4 loss, this mutant is also likely to be related to the +C mutants (the +C mutation in exon 4 might have caused this exon to be spliced out in this mutant) and it was considered so for further analysis of the mutation frequency.

Based on the sequence analysis of the TCR-γ gene rearrangement, 14 out of 15 analyzed mutants from sample 3E-0 were clonal with a +C frameshift following position

Table 3.3 Sequences of the TCRγ-gene rearrangements from pre- and post-treatment *hprt* mutants selected from SCLC patients undergoing etoposide chemotherapy[1].

Mutant ID	V gene	Vγ-gene sequence	N region	Jγ-gene sequence
7E4--10	V4	ACCT	CGGATGAGGAG	RAGAMWCTCTTY
7E3-2	V2	ACCTGG	ATGAACAACATCC	AAGAAACTCTTT
7E4-016	V8	ACC	CC	TTATTATGRAGAAACTCTTY
7E4-04	V2	ACCTGGGAYGGG	MMC	TTATTWTATAGRGAACYHYHHT
7E4--20	V8	ACCTKGGAT	CCCCTGCGG	TATTATAAGAAACTCHHY
7E2-12	V2/V8	ACCTGGGACGG	ACC (mixture)	GAATTAAGTATMAGAHACNCWNT
7E3-20	V7	ACCTGGGAC	GGACTT	TTATTATAAGAAACHCHHY
7E3-23	V8	ACCTSKGGATA	CATG	TTATAAGARACTCBNY
7E2-15	V8	ACCTGGGATA	CATG	TTATAAGAAACTCHNT
7E2-32	V8	ACCTGGGATA	CATG	TTATAAGAAACTCTNT
3E4-88	V8	ACCTGGGATA	CATG	TTATAAGAAACTCTHT
3E0-120	V8	ACCBCCCGAKA	MATC	KKKAKKWACSMAACY
3E0-77	V8	ACCYGGGATA	CATG	TTATAAGRAACTCYYT
3E0-38	V8	ACCTKGGATA	CATG	TTATAAGAAACTCNHB
3E0-82	V8	ACCTGGGGATA	CATG	TTATAAGAAACTCTYT
3E0-56	V8	ACCTGGGGATA	CATG	TTATAAGAAACTCYHH
3E0-74	V8	ACCYGGGATA	CATG	TTATAAGAAACTCTYT

[1] A-adenine, C-cytosine, G- guanine, T-thymine. All other letters were called by the computer sequence analysis software at places in the sequence with ambiguous base.

329 and, thus, only 2 mutants were counted as independent mutations. Assuming there was the same proportion of clonal mutants in the whole population of 148 mutants recovered from sample 3E-0, the mutant frequency ($958x10^{-6}$) was adjusted for clonality and the frequency of independent mutations became $63x10^{-6}$ (Table 3.1), which is within the MF range found in pre-treatment samples from other SCLC patients (Chapter II). Thus, it appears that the high MF values obtained for samples from patient 3E are due to the *in vivo* clonal proliferation of mutant with +C frameshift mutation. The MF adjusted for clonality (mutation frequency) becomes within the range of *hprt* MFs in a group of other SCLC patients (Chapter II). Moreover, sequence of the TCRγ-gene from the pre-treatment mutants with a +C frameshift was identical to that in one of the +C mutants from post-treatment sample 3E-4 suggesting that +C mutants selected in the pre- and post-treatment samples belong to the same *in vivo* proliferating T-cell clone (Table 3.3). Therefore, the +C clone persisted in patient 3E for at least 4 months.

Sample 3E-2. The fact that the MF in sample 3E-2 is substantially lower than that in other samples from this patient ($25x10^{-6}$) is interesting (Table 3.1). It appears that there are no clonal +C mutants in this sample. If this observation is correct, a possible explanation is that the etoposide treatment might have suppressed or eliminated a proliferating clone, as etoposide is most effective in killing dividing cells. It was difficult, however, to prove or deny this speculation. It was discovered that two of the five sequenced mutants selected from the post-treatment sample from another patient (sample 7E-2) also had a +C frameshift after position 329 (Table 3.2). Moreover, these two mutants (7E2-15 and 7E2-32) had the same TCR γ-gene rearrangement as the +C mutant

clone found in patient 3E as was evident from the sequence analysis (Table 3.3). This led to the suspicion that somehow cells from sample 7E-2 got mixed with cells from 3E patient samples, as it is very unlikely to find two T-cells in two subjects with the same *hprt* mutation and the same rearrangement of the TCRγ-gene. It was noticed that samples 3E-2 and 7E-2 were collected from the patients and delivered from the hospital to the laboratory on the same day (Table 3.1). These samples, each containing 4 Leucoprep tubes, were processed at the same time and it is possible that the tubes were mislabeled and got mixed among each other or that other mistake has occurred which allowed the mixing of cells between the two samples. Although it is not known how many tubes were mislabeled and what proportion of foreign blood was present in samples 3E-2 and 7E-2, the CE (14%) and MF ($154x10^{-6}$) determined for sample 7E-2 do seem to be higher than those values obtained during the same experiment with other cryopreserved samples from patient 7E (CE range is 4-9% and MF range is $36\text{-}98x10^{-6}$) (Table 3.1). At the same time, the MF in sample 7E-2 (154x10-6) is not as high as the MF values obtained for other cryopreserved samples from patient 3E (MF range is $546\text{-}958x10^{-6}$) (Table 3.1). Therefore, due to uncertainty in their identity, samples 3E-2 and 7E-2 were excluded from further analysis of MF and mutational spectra as the data obtained from these samples is unreliable (Chapter II).

It was also found that one of the mutants from another sample from patient 7E (mutant 7E3-23) also had the same TCRγ-gene rearrangement as mutants from the +C clone from patient 3E (Table 3.3). Mutant 7E3-23 had no +C frameshift mutation in exon 4. It was found to be a splice mutant with exon 5 loss (Table 3.4). As samples 7E-3 and 3E

Table 3.4 List of sequenced mutants from post-treatment samples from patient 7E and pre- and post-treatment samples from patient 1E.

Mutant ID	Mutation	Position	Exon	AA change	cDNA alteration	Sequence	Comments
7E3 (20)							
7E3-19	COM T>AC	23	1			GGC GTC GTG	created direct repeat TGGCGACC
7E3-13	TV T>G	571	8	Tyr->Asp		GGA TAT GCC	
7E3-12	TV T>G	203	3	Leu->Arg		GTG CTC AAG	
7E3-11	TV A>T	611	9	His->Leu		ttatagCAT GTT	
7E3-4	TS G>A	539	8	Gly->Glu		GTT GGA TTT	
7E3-20	TS G>A	544	8		exon 8 loss in some	TTT GAA ATT	cDNA of 2 bands
7E3-32	SU				exon 5 loss		
7E3-2	SU				exon 5 loss, exon 6 loss in some		2 cell pellets of 5 @ 1 bands
7E3-23	SU				exon 5 loss, exon 2-3 or 2-6 loss in some		2 cell pellets of 3 @ 3 bands
7E3-31	SU				21 bp of exon 8 loss	..TT GTT..CCA GAC	
7E4- (14)							
7E4--13	TV G>C	135	3	Arg->Ser		ctgtagG ACT GAA	
7E4--7	TV A>T	642	9	no change	exon 2@3 loss in some	AAA GCA AAA	cDNA of 3 bands
7E4--20	TS G>A		I7:-1		exon 8 or 21 bp of exon 8 loss	ctttttag TT GTT..CCA GAC	cDNA of 3 bands
7E4--15	SU				exon 8 loss, exons 2-3 or 6 loss in some		cDNA of 8 bands
7E4--10	SU				exon 8 loss		
7E4 (5 +27)							
7E4-08	TV G>C	3	1	Met->Ile		tccgttATG GCG	
7E4-01	TV T>G	392	5	Leu->Trp		GTC TTG ATT	
7E4-4	SU				exon 5 loss		

7E4-04	SU				exon 5 loss		2 cell pellets of 3@ 3 bands
7E4-019	SU				exon 6 loss		
7E4-02	SU				exon 8 loss		2 cell pellets of 1@ 3 bands
7E4-016	SU				exon 8 loss		
1E0 (5)							
1E0-1	F -A	263	3			AGA AAT AGT	
1E0-3	TS G>A	27	1	no change		GTC GTGgtgagc	
1E0-5	TS G>A	173	3	Gly->Glu		ATG GGA GGC	
1E0-4	SU				exon 4 loss		
1E0-2	SU				17 bp of exon 9 loss	....ATT AGT GAA ACT	cDNA of 2 bands
1E2 (17)							
1E2-6	F +T	531'	7'			CCA GAC T'gtaagtgaa	
1E2-1	TV A>T	131	2	Asp->Val		ATG GAC Aggtaa	
1E2-2	TV T>G	449	6	Val->Gly		TTG GTC AGG	
1E2-4	TV T->A	158	3	Val->Glu		GAT GTG ATG	
1E2-3	TS G>A	212	3	Gly->Asp		GGG GGC TAT	
1E2-5	TS G>A	606	8	no change		GAT TTG AATgtaagta	
1E2-10	SU				exon 2 loss, exon 2@3 loss in some		cDNA of 3 bands, no wt size
1E2-7	SU				exon 4 loss		
1E2-8	SU				exon 4 loss		
1E2-9	SU				exon 7 loss		

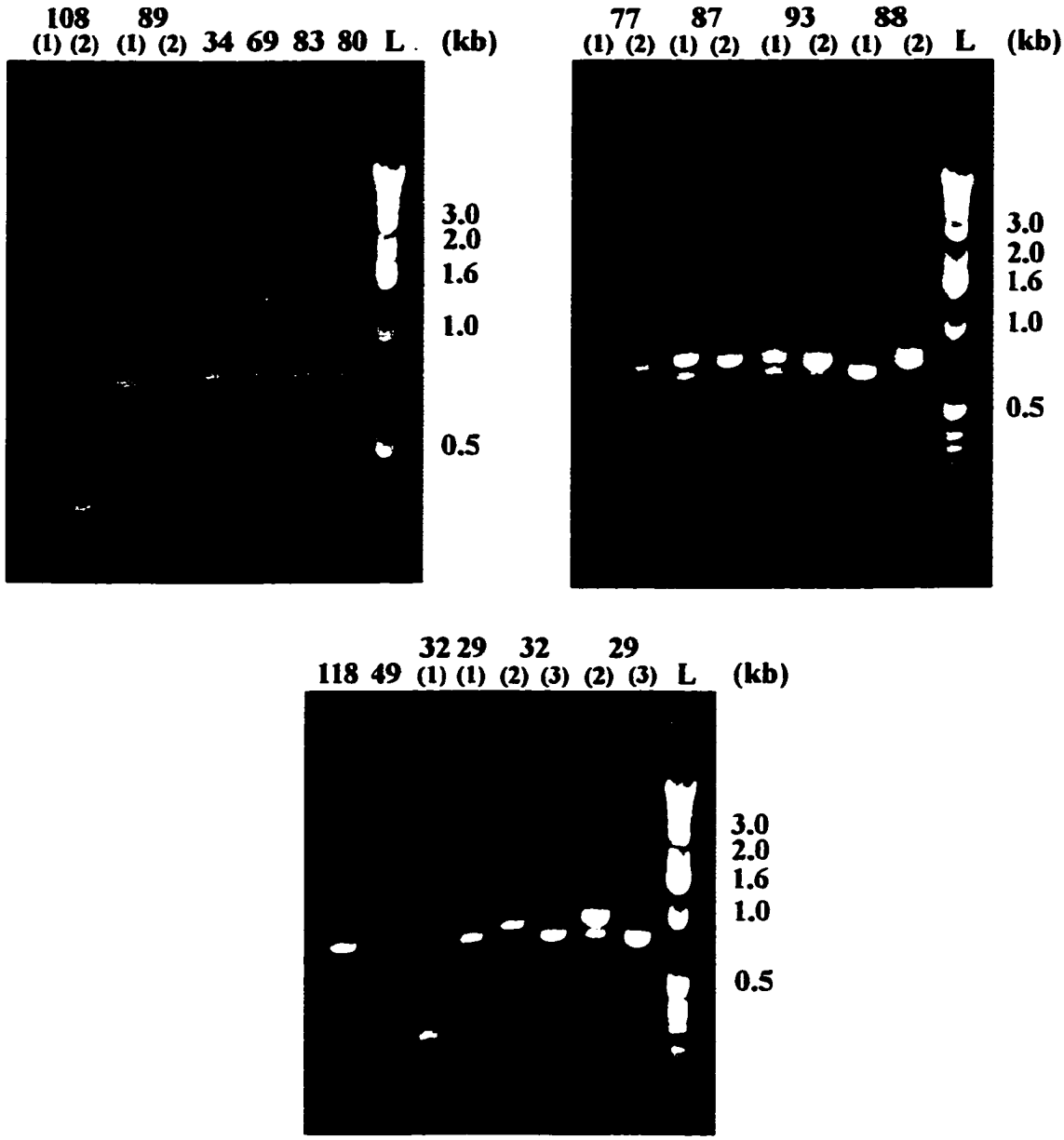


Figure 3.2

RT-PCR- amplified *hprt* cDNAs from *hprt* mutants selected from sample 3E-4 (mutant number is shown on the top of the gel). RT-PCR reactions from three individual cell pellets are shown for mutants 29 and 32 and RT-PCR reactions from two individual cell pellets are shown for mutants 77, 87, 93, 88, 108 and 89. L, 1kb DNA ladder (GibcoBRL).

samples were processed on different days, the possibility that the cells from these samples could have been mixed is excluded (Table 3.1). It appears that two *hprt* mutants were found from two different patients, which have identical rearrangement of their TCRγ-gene.

Multiple mutations? An observation was made with mutants from sample 3E-4 that the products of PCR amplification of reverse transcribed mutant *hprt* cDNAs contained multiple bands which could be resolved by electrophoresis and were seen on agarose gel runs (Figure 3.2). Moreover, when two or more different cell pellets from one mutant were used in the same RT-PCR reaction, the cell pellets often gave PCR products with different band patterns (some examples of such mutants are shown on Figure 3.2). Such events are common in the study of *hprt* mutants and are not specific to mutants from sample 3E-4. However, the number of mutants with multiple bands was higher in the collection of 3E-4 mutants and these mutants were used in an attempt to explain the appearance of multiple bands. Moreover, as most of the 3E-4 mutants were descendants from a single clone with a +C frameshift mutation, appearance of multiple bands and different band patterns suggested that these mutants might have various additional mutations causing alternative cDNA splicing. PCR products from each individual cell pellet were sequenced and the differences between the cell pellets and between various bands were identified.

Two mutants gave different band pattern in each of three cell pellets tested (Table 3.4). Mutant 3E4-32 had 3 PCR bands in the first pellet and just one PCR band, but of different size, in the other two pellets (Figure 3.2). Sequencing of the PCR products from

Table 3.5 Mutants from sample 3E-4 with multiple band RT-PCR products and "multiple mutations" found in the proportion of their cDNA molecules or cells. Asterisks indicate GC>AT transitions which as indicative of RT mistakes.

Mutant ID	Expanded "+C" clone Yes/No	Cell pellet/ No. of PCR bands	Cell pellet number in brackets and corresponding cDNA alteration	Mutation	Position	Exon	AA change	Sequence
3E4-32	Yes	1/3	(1) ex 2-6 loss/full with +C	F +C	329'	4		CAG TCA ACA
		2/1	(2) +C and 80 bp of I5 insertion		33858-33937			tagtacaa....catcaag...caggttg
			with TS	TS* C>T	33893			
		3/1	(3) +C					
3E4-29	Yes	1/1	(1) +C	F +C	329'	4		CAG TCA ACA
		2/2	(2) +C and I7 insertion					
		3/1	(3) +C and TS*	TS* G>A	173	3	Gly>Glu	ATG GGA GGC
3E4-108	Yes	1/1	(1) +C	F +C	329'	4		CAG TCA ACA
		2/3	(2) ex 2-5 loss/full length +C					
3E4-89	Yes	1/4	(1) +C	F +C	329'	4		CAG TCA ACA
		2/2	(2) +C					
3E4-77	No	1/1	(1) TS	TS G>A	212	3	Gly>Asp	GGG GGC TAT
		2/3	(2) ex 2-3 loss/full length +TS					
3E4-87	Yes	1/1	(1) +C	F +C	329'	4		CAG TCA ACA
		2/2	(2) exon 4 loss/full length					
3E4-88	Yes	1/1	(1) +C	F +C	329'	4		CAG TCA ACA
		2/2	(2) exon 4 loss					
3E4-93	Yes	1/2	(1) ex 4 loss/full length no TS					
		2/2	(2) TS*	TS* G>A	212	3	Gly>Asp	GGG GGC TAT
3E4-40	No	1/1		TS T>C	618	9	no change	GTT TGT GTC,
				TV T>G	299	3	Ile>Ser	TTT ATC AGA
3E4-111	Yes	1/1	exon 4 loss	TS* G>A	501	7	no change	AAA AGG ACC,
				TS* G>A	580	8	Asp>Asn	CTT GAC TAT
3E4-85	No	1/2	ex 2-6 loss/full length + 67 bp of I5	TV T>G	I5:+2			GAA gtaa..aaaggtaa
3E4-34	Yes	1/2	ex 4 loss/full length					

each of the three cell pellets revealed that the first pellet with 3 bands had a mixture of cDNA species with exons 2-6 loss (the shortest band) and a full length cDNA with a +C frameshift (the longest band) (Table 3.5). Although the sequence of the third middle band was not identified, it probably represents a cDNA with one exon loss (Figure 3.2). The second cell pellet had a cDNA with a +C frameshift and an 80 bp insertion of the middle of intron 5 with a GC>AT transition in the insertion (Table 3.5). The insertion accounts for the larger than normal size of the PCR amplified *hprt* cDNA obtained in this band (Figure 3.2). The last cell pellet had a normal size cDNA with a +C frameshift following position 329 (Table 3.5 and Figure 3.2). Another mutant, 3E4-29, also had three different band patterns in each of three cell pellets analyzed (Figure 3.2). In the first pellet with one band of normal size a +C frameshift was found (Table 3.5). The second pellet had two bands, one normal size and the other larger one (Figure 3.2). The sequencing of the product from the second pellet revealed a cDNA with a +C frameshift and an insertion of the entire intron 7 between exons 7 and 8 (Table 3.5). The last cell pellet from mutant 3E4-29 had a band of a normal size cDNA and the sequencing of this band revealed, that in addition to a +C frameshift in exon 4, there was a GC>AT transition at position 173 in exon 3 (Figure 3.2 and Table 3.5). Because this transition was found only in one out of three cell pellets from mutant 3E4-29, it is probably not a true mutation, but, rather, an RT artifact (see below).

Five other mutants each had two cell pellets with different number and patterns of their PCR bands (Figure 3.2). Mutant 3E4-108 had one band in the first cell pellet and three bands in the second pellet. The band in the first pellet and one of the bands in the second pellet with a normal size cDNA both had +C frameshift (Table 3.5). The shortest

band in the second pellet had a cDNA with exons 2-5 loss and the middle band in the second pellet probably corresponds to a cDNA with one or two exons missing (Table 3.5). The first cell pellet of mutant 3E4-89 had 4 bands while the second cell pellet had only 2 bands (Figure 3.2). Sequencing revealed the presence of only +C frameshift in both pellets (Table 3.3). The shorter size bands probably represent alternatively spliced cDNA species such as exon 4 loss and other spliced out multiple exons (Figure 3.2) Mutant 3E4-77 had one normal size band in its first cell pellet and 3 bands in the second cell pellet (Figure 3.2). Sequencing of the band in the first cell pellet revealed a GC>AT transition at position 212 while sequencing of the product from the second cell pellet revealed a mixture of full size cDNA and a cDNA with exons 2-3 loss (the shortest of the three bands) both with a GC>AT transition at position 212 (Table 3.3). The middle band in the second cell pellet is probably a cDNA missing one exon, most likely exon 2 (Figure 3.2). Mutants 3E4-87 and 3E4-88 each had two bands in one of the cell pellets and one normal size band in the other cell pellet (Figure 3.2). Sequencing revealed the presence of a +C frameshift in the normal size bands (Table 3.5). While a mixture of a full size cDNA and a cDNA missing exon 4 was detected in the double band PCR product of mutant 3E4-87, only one product with cDNA missing exon 4 was detected in the double band PCR product of mutant 3E4-88 (Table 3.5). The absence of a mixture of cDNA populations in the cell pellet with two bands in mutant 3E4-88 is likely due to the fact that the shorter of the two bands in this mutant was much stronger than the normal size PCR product and that might have resulted in it being preferentially sequenced (Figure 3.2).

Mutant 3E4-93 had double bands in both cell pellets, however, the normal size band in the second pellet was much stronger than the smaller size band (Figure 3.2). Sequencing revealed a mixture of a full size cDNA and a cDNA with exon 4 loss in the first pellet. A GC>AT transition at position 212 was the only cDNA alteration in the second cell pellet (Table 3.5). Again, like in mutant 3E4-29, because the transition appeared only in one of the cell pellets, it is probably not a mutation but an RT artifact (see below). On the other hand, contrary to mutant 3E34-29, where GC>AT transition was found in addition to the real mutation (+C frameshift), no +C frameshift or any other mutation was found in the second cell pellet from mutant 3E4-93. RFLP analysis showed that mutant 3E4-93 belongs to the expanded clone with a +C frameshift (see above). Because no +C frameshift was identified in this mutant, it is unclear what mutation caused the splicing out of exon 4 in the proportion of cDNA molecules. Mutant 3E4-29 together with mutant 3E4-69 were the only two mutants with no apparent +C frameshift mutation and yet belonging to the expanded +C clone as was evidenced from the analysis of their TCR-γ gene rearrangements (Table 3.2, Figure 3.1).

In a collection of 3E-4 mutants there were two mutants (3E4-40 and 3E4-111) with two base substitutions each, however, one of the substitutions did not result in the amino acid change (Table 3.5). Mutant 3E4-111 also had an exon 4 missing from its cDNA. Whether these double base substitutions were real mutations or RT artifacts (see below) is not known because only one cell pellet from each of these mutants was tested. Two other mutants (3E4-85 and 3E4-34) each had double band cDNA products (Figure 3.4 and data not shown). Mutant 3E4-34 had a mixture of a full-length cDNA and a cDNA

with exon 4 loss (Table 3.5). Mutant 3E4-85 had a mixture of a cDNA with a 67 bp of intron 5 insertion (terminating at the cryptic AG splice donor site) due to a TA>GC transversion in the splice donor site of intron 5 and a cDNA with exons 2-6 loss (Table 3.5).

Thus, 12 out of 20 sequenced mutants from sample 3E-4 had at least one of the analyzed cell pellets with either more than one *hprt* cDNA PCR product or an *hprt* cDNA with more than one identified mutation (Tables 3.2 and 3.5).

Patient 22E. Another clonal population of *hprt* mutants was found in sample 22E-4. Out of 14 mutants sequenced from this sample, 8 were found to have the same deletion of 11bp in exon 2 flanked by 6 bp repeats (Table 3.6). Several attempts to amplify the TCRγ-gene rearrangement from the cell lysates of these mutants using primers V1, V2 and J1, J2 failed (data not shown). Genomic DNA was isolated from mutant 22E4-4 and the PCR amplification using the same primers failed again. Then another primer, V9, was designed which aligns to V9, a variable gene rarely used in the TCRγ-gene rearrangements. Together with J1 primer, positive PCR product amplifying the TCRγ-gene rearrangement was obtained for mutants 22E4-4 and 22E4-10 (data not shown). The PCR band with TCR rearrangement from mutant 22E4-4 was also sequenced and the sequence of V9 was identified (data not shown).

Assuming the clonal relationship of mutants with the identical 11bp deletion, the MF obtained for sample 22E-4 ($34x10^{-6}$) was corrected for clonality and the corrected MF became 20x10-6 (Table 3.1). Unfortunately, only one mutant from sample 22E-0 was sequenced (cell pellets were made only for 4 out of 13 selected mutants and only 2 out of

Table 3.6 Sequenced *hprt* mutants selected from pre-treatment (22E-0) and post-treatment (22E-4) samples from patient 22E undergoing etoposide chemotherapy. Number in brackets indicates the total number of selected mutants from a sample. (Abbreviations are as in Table 2.6).

Mutant ID	Mutation	Position	Exon	AA change	Sequence
22E-0 (13)					
22E0-5	TS G>A	197	3	Cys>Tyr	CTC TGT GTG
22E-4 (17)					
22E4-3	D 3 bp	623-625	9		GTC ATT AGT GAA
22E4-8	D 11bp	116-126	2		CAT GGA CTA ATT ATG GAC A
22E4-4	D 11bp	116-126	2		CAT GGA CTA ATT ATG GAC A
22E4-15	D 11bp	116-126	2		CAT GGA CTA ATT ATG GAC A
22E4-9	D 11bp	116-126	2		CAT GGA CTA ATT ATG GAC A
22E4-13	D 11bp	116-126	2		CAT GGA CTA ATT ATG GAC A
22E4-10	D 11bp	116-126	2		CAT GGA CTA ATT ATG GAC A
22E4-14	D 11 bp	116-126	2		CAT GGA CTA ATT ATG GAC A
22E4-1	D 11 bp	116-126	2		CAT GGA CTA ATT ATG GAC A
22E4-5	F -T	75	2		ATA CCT AAT
22E4-2	TV C>A	550	8	Pro>Thr	ATT CCA GAC
22E4-16	TV T>G	194	3	Leu>Arg	GCC CTC TGT
22E4-12	TV A>T	133	2	Arg>Trp	ATG GAC AGgtaagtaag
22E4-7	NF				

4 mutants gave cDNA) and it is not known whether the same clone was present in a pre-treatment sample from this patient.

Clonality of mutants with the same splice errors from the same and repeat blood samples. In the cases when two *hprt* mutants from the same blood sample are found to have the same mutation, which is not at the mutational hot spot of spontaneous or induced mutagenesis, it is likely that these mutants are clonally related. The situation is, however, different when the mutants have identical splice errors such as loss of a common exon, because various mutations at various positions can lead to the splice errors, such as mutations at the splice sites or exon mutations. It is, therefore, much less likely that the mutants from the same blood sample with identical splice errors would be clonally related. To confirm this hypothesis the clonality of mutants derived from the same blood samples with the identical splice errors was investigated using the RFLP and/or sequencing of the TCRγ-gene rearrangements. Moreover, the clonality of mutants with the same splice errors derived from repeat blood samples from the same patient was investigated to determine whether mutants from the same clone could be picked up from the repeat blood samples.

Several mutants from either the same or repeat post-treatment samples from patient 7E had splice errors, which were either exon 5 or exon 8 losses (Table 3.4). Thus, mutants 7E2-12, 7E3-20, 7E3-31, 7E4--15, 7E4--20, 7E4--10, 7E4-02 and 7E4-016 all had partial or whole exon 8 loss in all or some of their cDNA molecules or cells (Table 3.4). At the same time, mutants 7E3-2, 7E3-23, 7E3-32, 7E4-4 and 7E4-4 all had exon 5 loss (Table 3.4). Results from the RFLP analysis of the TCRγ-gene rearrangements in mutants 7E3-2,

7E3-23 and 7E4-04 showed the clonal independence of mutants 7E3-2 and 7E3-23 as evidenced from the different patterns of restriction bands (Figure 3.3). Closer analysis of the band sizes revealed that V2 variable gene was used in the rearrangement in mutant 7E3-2, while V8 variable gene was used in the rearrangement in mutant 7E3-23 (Figure 3.3). Variable gene V2 was used in the rearrangement in mutant 7E4-04 and it was clearly clonally independent from mutant 7E3-23. The poor intensity of the bands in mutant 7E4-04 did not allow the identification of the crucial 42 bp band after the digestion with BstN1 enzyme and it was not possible to resolve the clonal relationship between this mutant and mutant 7E3-2 where V2 variable gene was also used in the rearrangement (Figure 3.3). Sequencing method, on the other hand, not only confirmed that V2 gene was used in the rearrangements in both mutants 7E3-2 and 7E4-04 but also resolved the differences in the sequence at the VJ junction between these two mutants proving their clonal independence (Table 3.3). Therefore, not only two mutants from the sample 7E3 with the identical exon 5 loss splice error are clonally independent, but they both are also clonally independent from the mutant with exon 5 loss selected from the later sample 7E-4.

The RFLP analysis also showed that mutants 7E3-20 and 7E4—10 (former mutant had exon 8 loss in some of the cDNA molecules and a GC>AT transition in exon 8 and the later mutant had an exon 8 loss) are clonally independent (Table 3.4, Figure 3.3). The band pattern in mutant 7E3-20 after digestion with RsaI has a bright band of undigested DNA and just one band of approximately 200 bp. Such pattern does not correspond to one that is expected after the RsaI digestion of V2, V3, V4 or V8 genes (Figure 3.3, Figure 1.10). The band pattern after BstN1 digestion in mutant 7E3-20 is similar to the

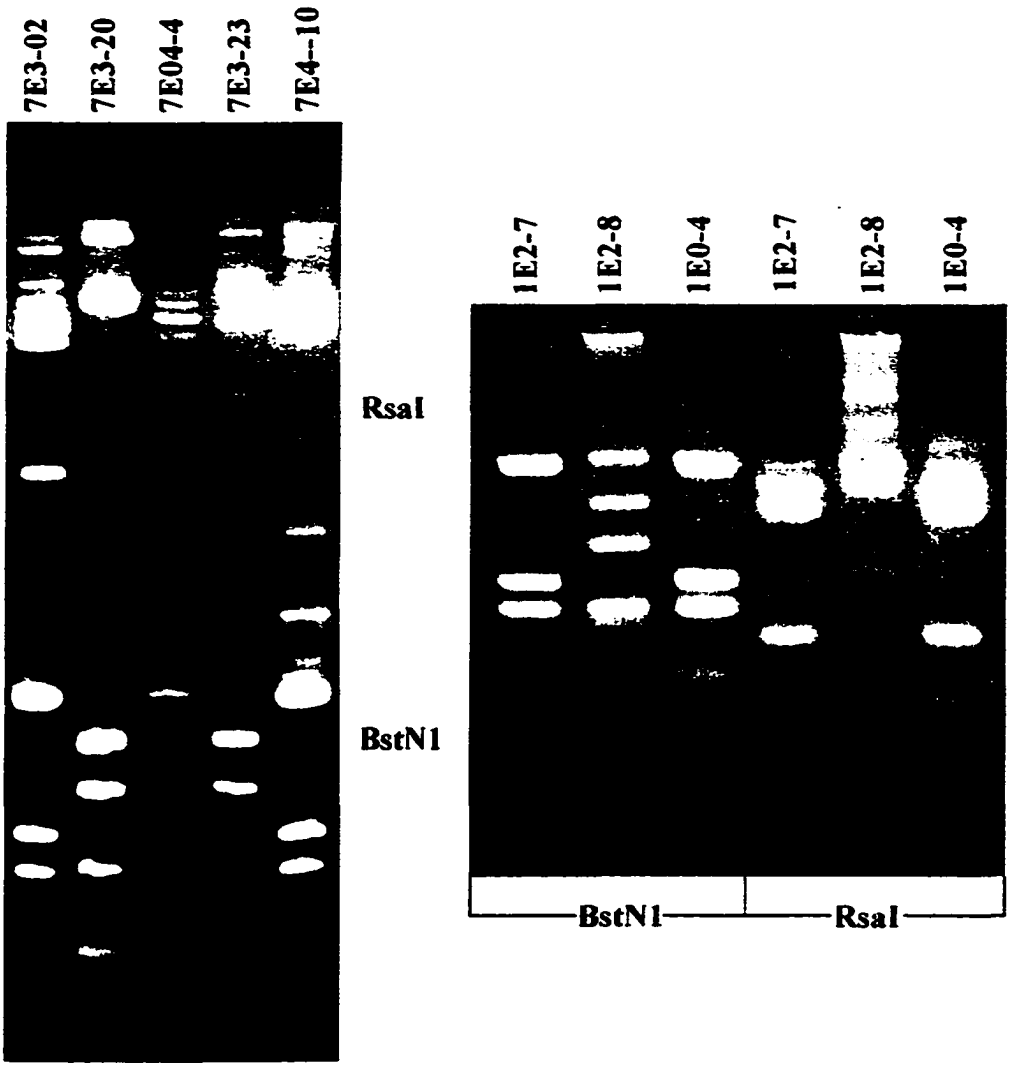


Figure 3.3 RFLP analysis of the TCR-γ gene rearrangements in *hprt* mutants selected from patients 7E and 1E (mutant number is shown on the top of the gel). PCR-amplified TCR-γ gene was digested with two restriction enzymes BstN1 and RsaI and fragments were separated on 5% NuSieve agarose gel.

pattern expected from the digestion of V8 gene. This situation is clearly different from the band patterns after digestion with both enzymes in mutant 7E4—10 that shows that V4 gene was used in the rearrangement (Figure 3.3). Sequencing method confirmed the use of V4 gene in the rearrangement in mutant 7E4—10, however, the use of a V7 pseudo-gene was found in the rearrangement in mutant 7E3-20 (Table 3.3). The unusual band pattern after RsaI digestion in mutant 7E3-20 can be explained by the use of the V7 gene in the rearrangement. Digestion of V7 gene might give the same pattern as the digestion of V8 gene with the BstN1 enzyme but not with the RsaI enzyme. Sequencing of the TCRγ-gene rearrangements in three other mutants with exon 8 losses (mutants 7E4-016, 7E4—20 and 7E2-12) revealed their clonal independence (Table 3.3). Contrary to mutants 7E4—10 or 7E3-20, where V4 and V7 genes were used in the rearrangements respectively, mutants 7E4—20 and 7E4-016 used V8 gene in the rearrangement, however, the sequences in the VJ junction or the N region were different in these mutants, indicating their clonal independence (Table 3.3). Mutant 7E2-12 was interesting in that the sequence of its TCRγ-gene rearrangement showed a mixture of two sequences belonging to V2 and V8 genes (Table 3.3). The ambiguous base was called at the positions where the sequence for V2 and V8 genes differed. Alternatively, the sequence was identical to the V2 gene sequence in some positions and to the V8 gene sequence in other positions (Table 3.3). The mixture of two variable genes in the rearrangement sequence may be explained by the mixture of two clones or, likely, by the use of different variable genes in the rearrangements of two alleles of the TCR γ-gene. The sequence at the VJ junction or the N region in mutant 7E2-12 clearly showed differences with N

region sequences from other mutants with exon 8 loss confirming the clonal independence of mutant 7E2-12 (Table 3.3). Therefore, all analyzed mutants with splice mutations leading to the whole or partial loss of exon 8 selected from the same or repeat blood samples from patient 7E were clonally independent.

Three mutants from patient 1E, all of which had exon 4 loss, were analyzed by the RFLP method to determine their clonality. Mutants 1E2-7 and 1E0-4 selected from the post-etoposide and pre-treatment samples respectively showed identical digestion patterns with both enzymes indicating their clonal relationship (Figure 3.3). The band patterns most closely resemble the patterns expected after digestion of V2 gene implicating its use in the TCRγ-gene rearrangements in mutants 1E2-7 and 1E0-4 (Figure 3.3). On the other hand, these band patterns were clearly different from those in mutant 1E2-8 where either V3 or V8 genes were used in the rearrangement (Figure 3.3). The appearance of extra bands on the BstN1 restriction digest in mutant 1E2-8 indicates either an incomplete digestion or possible mixture of V3 and V8 genes in the rearranged DNA (Figure 3.3). Thus, mutants from the same sample with the exon 4 loss in patient 1E are clonally independent, while mutants with the same splice error selected from the repeat samples are clonally related.

Automation of the sequencing method. To automate the sequencing method a new modification was proposed where sequencing reactions were done using only one dideoxyoligonucleotide, adenosine, for each DNA template. The products of sequencing reactions are then loaded respectively in A, C, G and T lane of the sequencing gel and are resolved by different colours. Thus, each color represents A's lane of the sequence for

each of 4 DNA templates from 4 different mutants. This method was tested with four mutants, two of which belong to the +C clone and two other mutants with alternative TCRγ-gene rearrangements. All 4 signals perfectly aligned up to the point where the junction between the variable and joining genes started (Figure 3.4). From this point, two colours (red and black) match perfectly representing clonally related T-cell clones but two other colours (blue and green) peak differently representing independent T-cell clones. The automation of the process permits quick analysis of a large number of mutants.

5. Discussion.

Patient 3E. MF and effect of etoposide treatment. The *hprt* MF was found to be highly elevated in the pre-treatment as well as three post-treatment samples from a SCLC patient receiving etoposide chemotherapy. An *in vivo* expanded *hprt* mutant clone was detected in two samples analyzed (samples 3E-0 and 3E-4) which can explain the unusually high MF values. When clonality was taken into account the corrected MF calculated for two samples with analyzed mutants in this patient became in the range of MF found in other age-matched SCLC patients receiving etoposide chemotherapy. Mutants from the expanded clone were found in the pre-treatment as well as post-treatment sample taken 4 months later indicating that the mutant clone persisted in the blood stream for at least 4 months and that etoposide treatment appeared to have no major effect on its persistence. Etoposide is a cell cycle phase-specific agent with maximum cytotoxicity in the S phase (Chow *et al.*, 1987). It would be, therefore, expected that *in vivo* proliferating *hprt* mutant T-cell clone would be highly susceptible to etoposide

Figure 3.4 Alignment of an adenine sequences for the V(D)J junction of the TCR-γ gene from 4 *hprt* mutants. Red and black, +C mutants from patients 3E; green and blue, mutants from other T cell clones. All 4 signals perfectly align up to the point where the junction between the variable and joining genes starts (Figure 3.4). From this point, two colours (red and black) match perfectly representing clonally related T-cell clones but two other colours (blue and green) peak differently representing independent T-cell clones.

killing. Persistence of the expanded clone in the circulation over 4 months period during several cycles of etoposide treatment indicates that either proliferating T-cells were not sensitive to etoposide treatment (became resistant) or that the expanded clone ceased to proliferate prior to etoposide exposure.

Although the MF in the sample taken after two etoposide cycles (sample 3E-2) was significantly lower ($25x10^{-6}$) than in all other samples from this patient, the possibility that a mislabeling mistake occurred resulting in the mixture of cells from sample 3E-2 with cells from other patient's sample was not excluded. No conclusion could be made on the changes in the MF or the effect of etoposide treatment on the MF in sample 3E-2.

The difference in MF in split sample 3E-4 when either fresh ($151x10^{-6}$) or cryopreserved cells ($765x10^{-6}$) were used in the experiment could in part be attributed to the difference in the CE which was higher for the fresh cells (23%) compared to the cryopreserved cells (15%). Other factors, such as cell death during freezing and variation in media conditions between two tissue culture experiments could have played a role in the discrepancy.

Patient 3E. Clonal mutants and their mutations. The finding of mutants with the same rearrangement of the TCRγ-gene as it is in the +C mutants, but without a +C frameshift mutation in sample 3E-4 is interesting. While no mutation was found in mutant 3E4-69, this mutant likely has a mutation outside the coding sequence which conferred its 6TG-resistant phenotype. Presence of such a mutant might indicate that clonal expansion of the +C clone started prior to the acquisition of a +C mutation by one of the cells in the clone. Other *hprt* mutations could thus be induced in the cells from this clone independent of the

+C frameshift. The same is true for mutants 3E4-85 and 3E4-93 that showed the rearrangements of their TCRγ-gene identical to the mutants from the +C clone, but had no detectable +C frameshift mutation. It is also possible, however, that the RFLP method is not sensitive enough to distinguish between minor differences in the N region of the TCRγ-gene rearrangements in these mutants.

The appearance of multiple bands in the products of PCR amplification of reverse transcribed cDNA is a common event in this study and was recorded in the literature (Chapters II and IV). Multiple bands are caused by the alternative splicing of exons in cDNA populations of a given cell pellet. Either mutations in the splice sites or exon mutations can cause the aberrant splice events. An interesting observation in this study was that such mutations resulted in different aberrant splice events in different cDNA molecules or cells in the cell pellet subjected to the RT-PCR reaction. Repeat RT-PCR reactions on different cell pellets from the same mutant resulted in alternative band patterns and an alternative cDNA splicing (Table 3.5). Each cell pellet contains approximately 2,000 cells and, as stimulated growing T-cells each may contain 6-9 *hprt* mRNA molecules (Steen *et al.*, 1991), each cell pellet subjected to RT-PCR reaction may contain up to 18,000 *hprt* mRNA molecules. Mutations in splice sites or exon mutations can lead to alternatively spliced mRNA molecules within the cell or between different cells in a cell pellet. Additionally, RT reaction and random PCR amplification can select for particular mRNA populations among variously spliced mRNA and cDNA molecules respectively. Combined, these factors contribute to the appearance of multiple bands. The

other reason for that may be the formation of hybrids between the full length cDNA and a cDNA lacking a particular exon(s).

The observation of several base substitutions in a single mutant is also a common event in this study and has also been recorded in the literature (Chapters II and IV, Curry *et al.*, 1997). Multiple base pair substitutions, particularly GC>AT transitions, have been explained by the mistakes introduced by the reverse-transcriptase enzyme (Curry *et al.*, 1997). It was found that the repetition of the RT step with a new cell pellet would eliminate multiple mutations in most cases (Curry *et al.*, 1997). This was also true for 3E-4 mutants where extra base substitutions would appear only in one but not in the other cell pellet such as in mutants 3E4-93 and 3E4-29 (Table 3.5). In the case of mutant 3E4-111, both GC>AT transitions are probably RT artifacts and the real mutation in this mutant is expected to be a +C frameshift after position 329 (TCR analysis showed that mutant 3E4-111 belongs to the expanded +C clone (Figure 3.1)). The frameshift could not be detected because exon 4 was lost from the cDNA in this mutant. In the case of mutant 3E4-40, the situation is less clear. Neither of the two base substitutions in this mutant are GC>AT transitions which is a typical base substitution introduced by the RT mistakes. However, one of the substitutions did not result in the amino acid change and should not confirm the mutant phenotype.

Although most of the "multiple mutations" seen in mutants from sample 3E-4 can be explained by an alternative mRNA splicing or RT mistakes, the proportion of such "multiple mutations" (>50%) is higher than it is normally detected in a mutant collection from a blood sample (Chapters II and IV). Nine out of 12 mutants with multiple band RT-PCR cDNAs or "multiple mutations" in sample 3E-4 belong to the detected *in vivo*

expanded clone and suspicion arose that the cells from these clone accumulated additional mutations during their *in vivo* expansion (Table 3.5 and Figure 3.1). The most likely candidates for such additional mutations are mutants 3E4-29 and 3E4-32 where an insertion of intron 7 and an insertion of 80 bp from intron 5 with a transition in the insertion were found respectively in addition to the +C frameshift (Table 3.5). No mutation in the splice sites of intron 7 was found in mutant 3E4-29, and it is not known what could have caused the insertion of 80 bp of the middle of intron 5 in mutant 3E4-32 (interestingly the insertion was flanked by the cryptic splice donor and acceptor sites as though the splice machinery recognized and inserted these 80 bp as an extra exon). Interestingly, these additional mutations were found only in a proportion of cells (or cDNA molecules) as some cell pellets from these mutants did not have the additional insertions (Table 3.5).

One possible explanation for the appearance of the alternative or multiple mutations in the expanded clone is that this clone became hypermutable with an increased rate of mutation. The additional mutations might have accumulated during *in vivo* and/or *in vitro* (during the assay) expansion of cells from the +C clone, which would explain mutations other than +C frameshift and mutations additional to the +C frameshift found in some cells from this clone. It appears that etoposide treatment might have had an effect on the evolution of the expanded clone.

Patient 3E. Mutational spectra and effect of etoposide treatment. Comparison of the mutational spectra in samples 3E-0 and 3E-4 indicates that mutants from the expanded clone are more prevalent in the pre-treatment sample (93% of sequenced mutants) than in the post-treatment sample taken 4 months later (60% of sequenced mutants) (Table 3.2).

This is consistent with a higher MF values obtained for the pre-treatment sample ($958x10^{-6}$ with cryopreserved cells) compared to the post-treatment sample ($151x10^{-6}$ with fresh cells or $765 x10^{-6}$ with frozen cells) (Table 3.1). The comparison also shows that expanded clone mutants from the post-treatment sample have more alternative splicing and multiple mutation events than do expanded clone mutants from the pre-treatment sample. Thus, 7 out of 12 (58%) +C mutants from sample 3E-4 had exon 4 loss at least in the proportion of the cDNA molecules or cells compared to only 2 out of 14 (14%) mutants with such losses in sample 3E-0 (Table 3.2). Also, two +C mutants from sample 3E-4 had additional insertions of intron sequence with no apparent mutations in the splice sites. Therefore, there is an indication that during the 4 month period of the etoposide treatment cells from this +C clone have accumulated additional *hprt* and/or other mutations or alterations that predispose them to have alternative exon splicing.

It remains unknown, however, whether etoposide treatment had any influence on the differences between the pre- and post-treatment mutational spectra in patient 3E. Etoposide, apart form being most cytotoxic in the S-phase cells, is also expected to be most mutagenic in S-phase and dividing cells due to the fact that DNA replication and cell division are necessary for DNA mutations to be fixed. As more than 90% of peripheral T-cells are in the G_0 phase of the cell cycle at any particular time, the *in vivo* expanding T-cell clone would seem to be the ideal target for the etoposide-induced mutagenesis. Etoposide is expected to induce large DNA deletions due to illegitimate recombination produced by its suppression of the DNA strand rejoining activity of topoisomerase II (Ferguson *et al.*, 1994). Although there was no indication of an increase in large deletions in the expanded clone, the prevalence of aberrant splice events and

possible multiple mutations in +C mutants from the post-treatment sample 3E-4 compared to the pre-treatment 3E-0 sample might be a secondary event to the etoposide exposure. Alternatively, lack of etoposide-induced large deletions in the post-treatment mutants may be due to the mentioned earlier possibility that the expanded clone ceased to proliferate prior to etoposide exposure.

Mutant clonality and its effect on the *hprt* MF. As it was mentioned earlier, lymphocyte populations *in vivo* contain uncommon large clones. When the *hprt* MF is determined in a number of individuals in a population, all measured MFs for individuals are being affected by discontinuous variation. Morley (1996) has determined that "the presence or absence of a large mutant clone in an individual results on the measured MF being either a large overestimate of the population MF in a minority of individuals or a small underestimate in the majority of individuals". Based on this observation, Morley concluded that standard MF and not MF adjusted for clonality should be used when a group of individuals is being used as a sample in order to estimate the MF of an underlying population (Morley *et al.*, 1996). The effect of variance from all sources, including that arising from the presence or absence of large clones, will be minimized by the fact that the sample consists of a number of individuals. Consistent with this, in the present study of groups of SCLC and MM patients undergoing chemotherapy standard MF were used for the determination of mean MF for the populations (Chapters II and IV). However, when an individual is being studied, the presence of a large clone leads to a substantial overestimation of the MF in this individual, as was the case in patient 3E. When the clonal analysis was performed and the MF was adjusted for clonality in this individual, the MF became in the normal expected range indicating that the overall

mutational load in that individual is comparable to that in other members of the same group of SCLC patients.

When two or more mutants are encountered bearing the same *hprt* mutation it is almost certain that they belong to the same expanded T cell clone. This was the case in mutants with identical +C frameshift in samples from patient 3E and mutants with identical 11 bp deletion in sample 22E-4. On the other hand, mutants with identical splice errors, such as exon loss events, are unlikely to be clonally related. This was demonstrated in mutants from patients 7E and 1E. The determination of the underlying mutation that led to the particular splice events in those mutants would likely reveal different mutational events at the splice sites or exons, which resulted in the same splice errors in those mutants.

This study also showed that mutants from the same clone can be recovered from the repeat blood samples (+C clone in patient 3E and exon 4 loss mutants in patient 1E). Screening of the repeat samples from an individual can, thus, be used to observe the evolution of the *in vivo* expanding clone to determine its life-span and possible accumulation of additional mutation. This is particularly relevant to cases with large mutant clone(s).

Comparison of RFLP and sequencing methods. Although the RFLP method is useful for the clonality detection, it appears that sequencing method is superior for the determination of the TCR-gene rearrangements. One of the drawbacks of the RFLP method is the appearance of extra bands after the restriction digests that can complicate the interpretation of the results. These bands can result from the uncompleted digest or from nonspecific PCR amplification. As it was shown in this study, extra bands can also

result from digests of mixtures of different variable genes or digests of alternative variable genes for which the restriction band patterns are unknown. Also, the slight difference in the 42 bp band in the BstN1 digestion, which spans the VJ junction and is crucial for the determination of mutant clonality in cases when the same variable genes were used in the rearrangement, may not be resolved by the agarose gel. The sequencing method, on the other hand, is more sensitive in that it allows one base pair resolution and T-cells with N region differing in only one bp can be distinguished. The sequencing method is less labor consuming, requires less DNA template and more informative. This method also allows for the determination of the variable gene used in the rearrangement and provides the exact sequence of the N region.

One of the interesting features found with the sequencing method was the identification of two different rearrangements within one T-cell clone (Table 3.3). This feature appeared as a mixture of two base pairs at positions where highly homologous variable genes differ in their sequence. Accordingly, the sequence of the N regions appeared as a mixture of two different sequences. One explanation for such mixtures is the fact that the TCR genes are rearranged on both alleles independently. VDJ recombinase rearranges randomly the variable and joining gene segments on both alleles and the finding of two different TCR gene rearrangements on two alleles is not uncommon. Out of 9 independent mutants analyzed by sequencing method, one was found to have a mixture (Table 3.3). While a substantial number of T-cells can have an asynchronous rearrangements of the TCR genes on their two alleles, studies have shown that only a small proportion of cells express two variable genes (approximately 1% of

human peripheral blood T- cells express two V_β genes) and the mechanism of allelic exclusion has been proposed to account for this (Padovan *et al.*, 1995). This mechanism provides further diversity to the T-cell specificity. The mixture of two rearrangements, however, should not prevent or complicate the identification of clonal mutants since the same mixtures of the rearrangements should appear in clonal mutants and independent mutants should have different mixtures if in both mutants their TCR-γ alleles were rearranged differently. Thus, mutant 7E2-12 was found to have a mixture of V2 and V8 gene sequences in its TCRγ-gene rearrangement, however, this did not prevent the identification of its clonal independence from other mutants with V2 or V8 rearranged genes (Table 3.3).

Another interesting feature found with the sequencing method in this study was that while some TCR-γ variable genes, such as V2, V3, V4 and V8, are preferentially used in the rearrangements, others such as rarely used V9 gene and silent pseudo-genes can also be rearranged. Thus, V9 gene was found in the rearrangement in the expanded clone in sample 22E-4 and variable pseudo-gene V7 was found in the rearrangement in mutant 7E3-20 (Table 3.3). It is important to emphasize that the appearance of more or less bands than that expected from the known patterns of the restriction digests of V2, V3, V4 and V8 genes can indicate that the variable genes other than V2, V3, V4 and V8 were used in the rearrangement. This was the case with mutant 7E3-20 where the pseudo-gene V7 likely produced unrecognizable band pattern after the RsaI digestion (Figure 3.3).

The last interesting feature found with sequencing method was the identification of two mutants selected from different patients with identical TCR-γ gene rearrangement

including the use of the same variable gene and the identical N region (Table 3.3). The TCR genes are rearranged during T-cell maturation in the thymus by the random action of VDJ recombinase. The possibility of finding T-cells with identical rearrangement of the TCR genes occurring by chance along in two different subjects is very low. The same situation was found in another study. Three *hprt* mutants selected from three individuals had identical TCR-γ gene rearrangements (John Curry, personal communication). It would appear thus, that such event is common, however, the mechanism and significance of it is unclear.

CHAPTER IV. *hprt* MUTANT FREQUENCY AND MUTATIONAL SPECTRA IN T-LYMPHOCYTES OF MULTIPLE MYELOMA PATIENTS RECEIVING MELPHALAN/PREDNISONE CHEMOTHERAPY

1. Abstract.

Melphalan, a bifunctional alkylating agent and a known mutagen, has been implicated as a causative agent of secondary acute myeloid leukemia. We have investigated the potential mutagenic effect of melphalan in cancer patients receiving melphalan/prednisone chemotherapy by using the *hprt* T-cell clonal assay. Nine multiple myeloma patients (mean age 69±9) received monthly doses of melphalan/prednisone chemotherapy (mean total dose of melphalan 443±192 mg) over the period of 4.3 to 23 months. The peripheral blood samples were collected before treatment and up to three times following the treatment cycles. Results revealed no significant increase in the mean *hprt* mutant frequency (MF) in patients after the treatment (mean MF= $47 \pm 37 \times 10^{-6}$ before treatment and $54 \pm 41 \times 10^{-6}$ after treatment). One patient, who underwent the most cycles and thus was followed for the longest time, showed a steady significant increase in the MF following chemotherapy cycles. The sequence analysis of 74 pre-melphalan and 89 post-melphalan *hprt* mutants revealed no dramatic differences between the spectra and the distribution of the different classes of mutations. The only significant difference between the pre- and post-treatment mutational spectra was in the proportion of GC>TA transversions. These were increased in post-melphalan spectra. The results suggest that melphalan chemotherapy is unlikely to be highly mutagenic. Alternatively, serious

consideration must be given to the possibility that this assay is not sensitive enough to detect the increase in the MF. The results also suggest that melphalan treatment can increase mutation in patients with prolonged exposure, and that GC>TA transversions might be induced by the melphalan chemotherapy which is consistent with the genotoxic damage by alkylating agent melphalan.

2. Introduction.

Melphalan is one of the most common nitrogen mustards used in chemotherapy treatment of various types of cancer including lymphomas, breast, ovarian cancer and multiple myeloma (Ferguson *et al.*, 1996). Although a useful agent, it is associated with a treatment-induced cancer risk. As many as 10-20% of cancer patients develop secondary acute myeloid leukemias (sAML) several years after treatment with nitrogen mustards (Povirk *et al.*, 1994, Bergsagel *et al.*, 1979, Rodjer *et al.*, 1990, Curtis *et al.*, 1984). The mechanism by which melphalan and other chemotherapy agents may induce secondary cancer is not well understood. There have been several studies of the effect of melphalan treatment on the chromosomal damage. Correlation between the types of translocations induced by melphalan *in vitro* and *in vivo* and the translocations found in sAML has been found (Mamuris *et al.*, 1989 (1), Mamuris *et al.*, 1989 (3), Mamuris *et al.*, 1990). Mutation induction in cancer patients by melphalan chemotherapy, however, has not been investigated.

DNA damage, mutagenicity and carcinogenicity of melphalan, a bifunctional alkylating agent, is well documented (Ferguson *et al.*, 1996, Povirk *et al.*, 1994). Melphalan induces monofunctional guanine-N7 and adenine-N3 adducts as well as intra- and interstrand crosslinks involving guanines and adenines. Interstrand crosslinks are

believed to be primarily responsible for cytotoxicity. Melphalan has been shown to induce a wide spectrum of mutations and the correlation has been found in some cases between the sites of potential DNA adducts and mutation. Thus, the predominance of mutations at AT base pairs, especially AT>TA transversions, has been found in melphalan-induced spectra in the SV40-based shuttle vector pZ189 system (Wang *et al.*, 1990). The sites of mutations were correlated with the sites of adenine-N3 adducts. In contrast, GC>TA and GC>CG transversions at the 5'G-N-C3' potential interstrand crosslink sites predominated the melphalan-induced spectra in the hemizygous *aprt* gene of CHO-D422 hamster cells (Austin *et al.*, 1992). In addition, melphalan has been shown to induce large, multilocus deletions in germ cells of the mouse (Russell *et al.*, 1992 (2)). Despite our understanding of melphalan's interaction with DNA, it remains unclear what kind of mutation melphalan produces in normal human cells.

There have been a number of studies of the mutagenicity of chemotherapy in cancer patients using the *hprt* T-cell clonal assay. Although results of these studies are often complicated by the use of multiple drugs, those regimens, which included alkylating agents, were associated with the increased frequency of the *hprt* mutation after treatment (Tates *et al.*, 1994, Ammenheuser *et al.*, 1988, Palmer *et al.*, 1988, Branda *et al.*, 1991, Caggana *et al.*, 1991). In this study the possible mutagenic consequences of melphalan/prednisone chemotherapy were investigated in multiple myeloma patients using this assay.

3. Materials and methods.

Patients and samples. The procedure for sample collection was described previously (Chapter III). The usual treatment regimen for this group of MM patients was monthly

chemotherapy cycles consisting of 24-72 mg of melphalan and 400 mg of glucocorticoid prednisone administered over a 4- day period. The blood samples were collected usually on the day the following treatment cycle began. The pre-treatment cycles were collected from each of the 9 patients (mean age 69±9) included in the study (Table 4.1). Selected mutants from three other patients were sequenced and included in the mutational spectra analysis. Nine patients were followed for 4.3 to 23 months (mean 10.3±1.8) from the beginning of the treatment and received a total melphalan dose of 186- 840 mg (mean 443±192) (Table 4.2). One melphalan patient (5M) received additional monthly cycles of another glucocorticoid dexamethasone (120 mg in 4 days) during the 3 months prior to the last blood sample (Table 4.2).

Tissue culture and the molecular analysis of mutants. The procedures for the isolation and molecular characterization of *hprt* mutants were described previously (Chapter III).

Sequencing. Sequencing of the *hprt* cDNA was performed on LI-COR sequencer (Li-COR Inc., Lincoln, NE) using SequiTherm EXCEL Long-Read DNA sequencing kits (Epicentre Technologies Corporation, Madison, WI) according to manufacturer's protocol. Two Fluorescent (IRD41)-labeled primers (LI-COR Inc., Lincoln, NE) A and E (Chapter III) were used for obtaining an overlapping sequence of the *hprt* cDNA. The mutant sequences were analyzed using SeqMan analysis program (DNASTAR Inc., Madison, WI).

Statistical analysis. Ln(MF)=f(age,CE) relationships were obtained using multiple regression analysis, the pre-(post)-treatment MF were compared using t-test and spectra comparisons were done using observed *vs.* expected χ^2 tests (Statistica, Tusla, OK).

Table 4.1. Information on 12 MM patients receiving melphalan/prednisone chemotherapy.

PATIENT ID	AGE (years)	SEX	SMOKING (1pack/day× years)
1M	66	F	none
2M	76	M	pipe for few years 35 years ago
3M	63	M	smoker till 0.5 years ago
4M	55	M	smoker
5M	68	F	8-10 cig/day 17-47 years old
6M	85	M	smoker till 25 years ago
7M	62	M	smoker till 20 years ago
9M	77	M	56
10M[a]	75	F	not known
14M	70	F	2cig[c]/day
15M[a]	77	F	none for 15 years
18M[a]	82	M	none for >20 years
range[b]	55-85		
mean[b]	69±9		

Cig, cigarettes.

[a]The pre-treatment samples from patients 10M and 15M as well as post-treatment samples from patient 18M were not available for analysis and, therefore, these patients were not included in the MF data analysis. However, selected mutants from other samples from these patients were sequenced and included in the mutational spectra analysis (Table 4.8).

[b] Values from patients 10M, 15M and 18M are not included.

Table 4.2. Timing of blood samples relative to the number of melphalan/prednisone treatment cycles and melphalan dose in MM patients.

Sample ID[d]	Timing since the first cycle (months)	Total previous doze (mg)	Number of previous cycles	Timing since the last cycle (months or days)
1M-0[d]				
1M-1	2.3	70	1	2.3 months
1M-2	10.3	500	9	1 day
2M-0[d]				
2M-1	9.1	296	2	2 months
2M-2	12.1	438	5	1.3 months
3M-0[d]				
3M-1	7.3	400	6	1 month
4M-0[d]				
4M-1	3.3	204	3	0.7 months
4M-2	8.3	408	6	1.6 months
5M-0[d]				
5M-1	4.7	280	5	3 days
5M-2	10.7	616	11	1 month
5M-3	15.7	728	13	3.8 months
5M-4	23	840	15[a]	5.7 months
6M-0[d]				
6M-1	6.5	110	4	1.2 months
6M-2	12	206	8	1 month
7M-0[d]				
7M-1	8.7	528	9	not known
9M-0[d]				
9M-1	2	192	2	0 days
9M-2	6.6	480	7	1 month
10M-1[b]	3.1	192	3	1 month
10M-2[b]	9.1	576	9	1 month
14M-0[d]				
14M-1	4.3	186	3	1 month
15M-2[b]	8.4	576	9	1 month
18M-0[b,d]				
range[c]	4.3-23	186-840	3-15	0 days-5.7 months
mean[c]	10.3±1.8	443±192	8±3	

[a] Patient 5M received additional 3 cycles of dexamethasone prior to the last blood sample.
[b] See footnote f to Table 4.1.
[c] The values from samples 10M-1, 10M-2, 15M-2 and 18M-0 were not included.
[d] Pre-treatment samples.

Table 4.3. Comparison of the pre- and post-treatment *hprt* data for the MM patients received melphalan/prednisone chemotherapy.

	pre-treatment				post-treatment[a]			
	N_0	CE(%)	10^{-6}xMF	ln(10^6xMF)	N_1	CE(%)	10^{-6}xMF	ln(10^6xMF)
range	9	1-17	13-132	2.6-4.9	17	2-20	9-152	2.2-5.0
median		9	33	3.5		6	36	3.6
mean		10	47	3.6		8	51	3.7
SD		5	37	0.7		5	37	0.7

[a] For each patient the means of the values after each treatment cycle were used. $N_0(_1)$ - the number of pre-(post)-treatment samples analyzed.

4. Results.

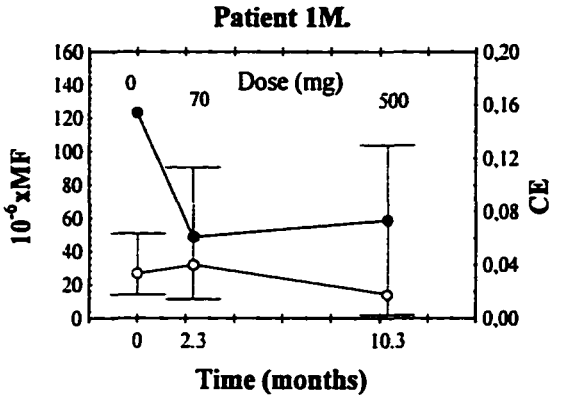
Mutant Frequency. There was no significant increase in mean MF in this group of MM patients after melphalan/prednisone chemotherapy (t=-0.2, p=0.9) (Table 4.3). There was no correlation between MF and the drug dose (R^2=0.04, p=0.35).

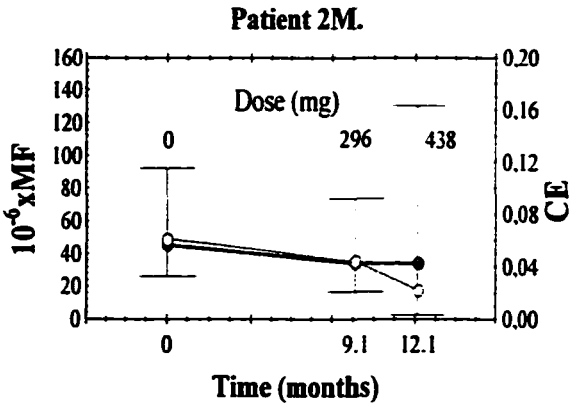
Inter-patient variability in response to chemotherapy was observed in respect to MF, which increased in 4 patients after treatment (Figure 4.1).

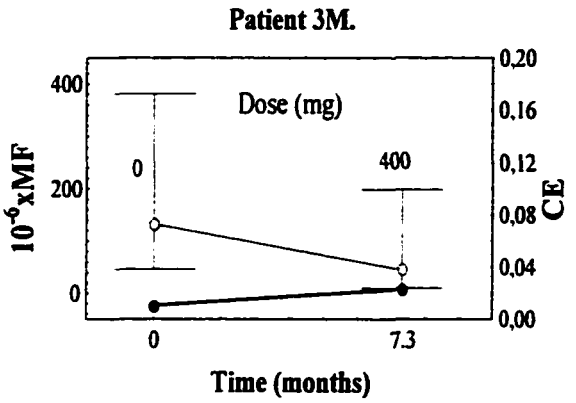
In only one patient (5M), an apparent dose related significant increase in MF was observed. A 7-fold increase in MF was seen at 15 and 23 months into the chemotherapy regimen (Figure 4.1). The last sample from this patient was taken 6 months after completion of the melphalan treatment with a total dose of 840 mg and post 3 cycles of a total of 480 mg of dexamethasone (Figure 4.1).

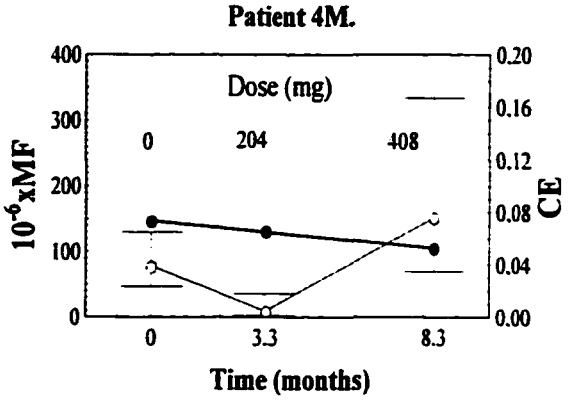
Lower than usual CE values were obtained from these samples with 17 out of 26 samples having CE of less that 10% (Table 4.4). There was an additional slight decrease in CE in the post-treatment samples (Table 4.3).

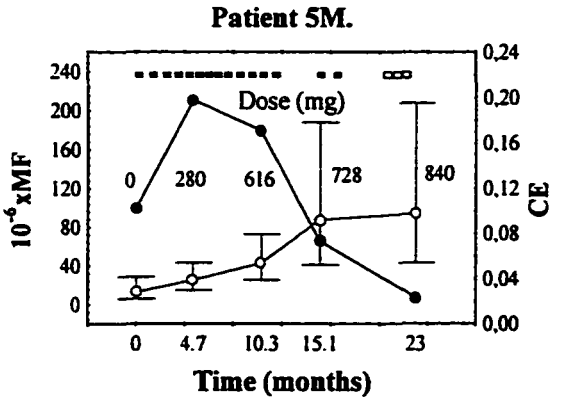
Figure 4.1. Results of the *hprt* T-cell clonal assay on CE and MF changes with time in 9 MM patients during melphalan/prednisone chemotherapy. Peripheral blood samples were obtained from the patients before treatment (time 0) and one or more times after melphalan cycles (other time points). Pre-stimulated T-cells were grown in the presence of interleukin 2, and hprt mutants were selected by the addition of 6-thioguanine to the medium. The MF for each sample was calculated based on the number of surviving colonies and was adjusted for the corresponding CE. °, MF; •, CE. 95% confidence intervals for MF were calculated based on CE and the original number of cells used (Branda *et al.*, 1993). The time course of melphalan/prednisone cycles for patient 5M is shown with closed rectangles, dexamethasone cycles are shown with open rectangles.

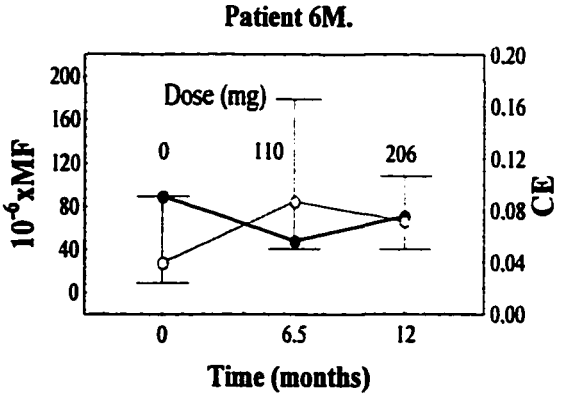

Patient 1M.
Dose (mg)
0
70
500
10^{-6}xMF
CE
Time (months)
0
2.3
10.3


Patient 2M.
Dose (mg)
0
296
438
10^{-6}xMF
CE
Time (months)
0
9.1
12.1

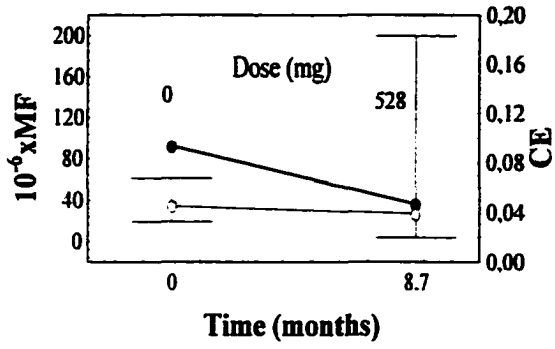

Patient 3M.
Dose (mg)
0
400
10^{-6}xMF
CE
Time (months)
0
7.3


Patient 4M.
Dose (mg)
0
204
408
10^{-6}xMF
CE
Time (months)
0
3.3
8.3

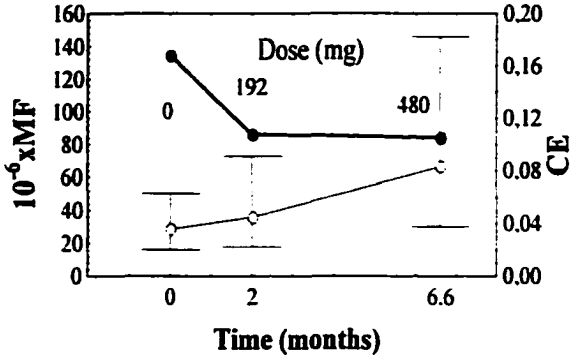

Patient 5M.
Dose (mg)
0
280
616
728
840
10^{-6}xMF
CE
Time (months)
0
4.7
10.3
15.1
23


Patient 6M.
Dose (mg)
0
110
206
10^{-6}xMF
CE
Time (months)
0
6.5
12

Patient 7M.



Patient 9M.



Patient 14M.

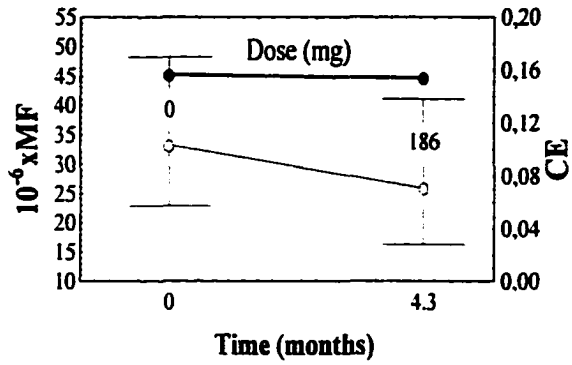


Table 4.4. Results of the *hprt* T-cell clonal assay in 12 MM patients receiving melphalan/prednisone chemotherapy.

Sample ID	Non-selective + wells	Non-selective total wells	Selective + wells	Selective total wells	CE	MF x10^{-6}	95%[d] confid.int. Upper level	95%[d] confid.int. Lower level	Fresh cells[a] Y/N
1M-0 (1)[b,c]	78	192	29	272	0.17	65	100	42	
1M-0 (2)[b,c]	71	192	11	272	0.15	27	51	14	
1M-1 (1)[b,c]	54	192	3	280	0.11	10	31	3	
1M-1 (2)[b,c]	32	192	4	208	0.06	32	90	11	
1M-2	38	192	1	96	0.07	14	104	2	
2M-0	30	192	14	512	0.06	49	92	26	
2M-1	23	192	10	676	0.04	35	74	17	
2M-2	23	192	1	134	0.04	18	130	2	Y
3M-0	6	192	8	576	0.01	132	381	46	
3M-1	13	192	2	192	0.02	45	199	10	
4M-0	38	192	24	432	0.07	78	130	47	
4M-1	34	192	2	360	0.06	9	36	2	
4M-2	28	192	8	104	0.05	152	334	69	
5M-0	51	192	7	528	0.10	13	29	6	
5M-1	86	192	16	328	0.20	25	43	15	
5M-2	77	192	17	240	0.17	43	73	25	
5M-3	38	192	8	128	0.07	88	188	41	
5M-4	13	192	12	548	0.02	95	208	43	
6M-0	46	192	3	120	0.09	28	89	9	
6M-1	30	192	9	192	0.06	85	179	40	
6M-2	39	192	26	536	0.08	66	108	40	Y
7M-0	47	192	15	480	0.09	34	61	19	
7M-1	25	192	1	80	0.05	27	200	4	Y
9M-0	76	192	14	300	0.17	28	50	16	
9M-1	53	192	9	238	0.11	36	73	18	
9M-2	52	192	7	104	0.11	66	146	30	
10M-1[c]	29	192	20	904	0.05	41	72	23	

Sample ID	Non-selective + wells	Non-selective total wells	Selective + wells	Selective total wells	CE	MF x10^{-6}	95%[d] confid.int. Upper level	95%[d] confid.int. Lower level	Fresh cells[a] Y/N
10M-2[c]	20	192	7	312	0.04	62	146	26	Y
14M-0	72	192	45	888	0.16	33	48	23	
14M-1	71	192	24	616	0.15	26	41	16	Y
15M-2[c]	4	192	3	400	0.01	107	479	24	Y
18M-0[c]	32	192	3	112	0.06	45	146	14	Y

[a] The last column indicates whether fresh or previously frozen cells were used in the experiment.
[b] Samples 1M-0 and 1M-1 were split and split samples were processed an different days. Samples 1M-0 (2) and 1M-1 (2) were processed on the same day and they were used in the MF data analysis (Table 4.2).
[c] The MF data from these samples was not included in the MF data analysis (Table 4.1)., Selected mutants from these samples were sequenced and included in the mutational spectra analysis (Table 4.8).
[d] 95% confidence intervals were calculated based on the CE and total number of cells used and according to Branda *et al.* (1993 (1)).

The lnMF=f(CE,age) relationships obtained for this sample collection were compared to the relationships obtained in the age-matched unexposed control population and in our previous study of etoposide chemotherapy (Karnaoukhova *et al.*, 1997) (Table 4.5). LnMF and CE were independent of age for all sample groups. However, contrary to all other sample groups, the lnMF was also independent of the CE in our melphalan study. This difference is probably an artifact and can likely be attributed to the relatively low CE values obtained for these samples as well as to the low sample number (Table 4.5). When all chemotherapy samples processed in our etoposide and melphalan studies were considered together, the lnMF=f(CE,age) relationships were close to those obtained for the unexposed control population (MF and CE are independent of age and lnMF is inversely proportional to CE (CE>5%)) (Table 4.5). The only difference is that our samples had lower CE and consequently higher MF (Table 4.5). The low CE may be related to the various techniques used by us and other laboratories as well as to the disease state and treatment of patients in these chemotherapy studies.

Mutational Spectra.

Mutant representation. RT-PCR was attempted on cell pellets from 232 mutants including 107 pre-treatment and 125 post-treatment mutants from a total of 32 samples (Table 4.6). These mutants included those selected from the samples included in the MF data (Table 4.4) as well as mutants selected from other samples not included in the MF data. The other samples include split samples 1M-0 (1) and 1M-1 (1) processed on different days, two post-treatment samples from patient 10M, one post-treatment sample from patient 15M and one -pre-treatment sample from patient 18M (Table 4.6).

Table 4.5. Comparison of lnMF = f (age,CE) relationships obtained in the current study with the relationships obtained for the age-matched unexposed control population (Curry *et al.*, submitted). N-number of subjects.

Source	Age[c]	N[a]	Relationship[b]	R^2 and p values
Curry *et al.*(submitted),	65-85	147	lnMF=3.3-0.01age	R^2=0.00008, p=0.3
(CE>5%)	70±5	148	lnMF=3.1-1.7CE	R^2=0.2, p=0.000000
		148	CE=0.3-0.0007age	R^2=0.0004, p=0.8
Melphalan study. All	55-85	9	lnMF=5.8-0.03age	R^2=0.17, p=0.3
samples.	69±9	26	lnMF=4.06-4.55CE	R^2=0.11, p=0.1
		9	CE=-0.1+0.002age	R^2=0.08, p=0.5
Melphalan study, CE>5%		8	lnMF=4.8-0.02age	R^2=0.12, p=0.4
		20	lnMF=4.07-4.4CE	R^2=0.08, p=0.2
		8	CE=0.06+0.0007age	R^2=0.025, p=0.4
Etoposide study. All	66-83	12	lnMF=3.57-0.0004age	R^2=0.000007, p=1.0
samples	75±6	31	lnMF=4.16-4.63CE	R^2=0.22, p=0.007
		12	CE=0.24-0.0017age	R^2=0.03, p=0.6
Etoposide study, CE>5%		10	lnMF=5.84-0.03age	R^2=0.09, p=0.4
		24	lnMF=3.46-0.94CE	R^2=0.01, p=0.6
		10	CE=0.08+0.0006age	R^2=0.006, p=0.8
All chemotherapy	55-85	21	lnMF=4.95-0.02age	R^2=0.04, p=0.4
samples.	72±8	57	lnMF=4.2-5.76CE	R^2=0.25, p=0.00009
		21	CE=0.07+0.0005age	R^2=0.005, p=0.8
All chemotherapy		18	lnMF=5.21-0.025age	R^2=0.102, p=0.2
samples, CE>5%		44	lnMF=3.9-3.71CE	R^2=0.11, p=0.03
		18	CE=0.06+0.0008age	R^2=0.021, p=0.6

[a] Outliers (cases for which the absolute standard residual value is more than 3) determined for each individual relationship were excluded.
[b] In lnMF=f(age) and CE=f(age) relationships in the current study only values from the pre-treatment samples were included.
[c] Range, mean ± SD.

Table 4.6. The number of sequenced and *hprt* cDNA negative mutants obtained after RT-PCR reaction on cell pellets from mutants selected from 32 samples in multiple myeloma patients.

Sample	Mutants Selected	Positive RT-PCR Mutants sequenced		Negative RT-PCR	
		N	(%)	N	(%)
1M-0(1)	29	5	17		
1M-0(2)	11	8	73		
1M-1(1)	3	3	100		
1M-1(2)	4	0	0		
1M-2	1	1	100		
2M-0	14	5	36		
2M-1	10	0	0		
2M-2	1	0	0		
3M-0	8	6	75		
3M-1	2	2	100		
4M-0	24	18	75	3	14
4M-1	2	0	0		
4M-2	8	4	50	2	33
5M-0	7	4	57	1	20
5M-1	16	8	50	2	20
5M-2	17	6	35	2	25
5M-3	8	1	13	1	50
5M-4	12	10	83	1	9
6M-0	3	2	67		
6M-1	9	9	100		
6M-2	26	25	96	1	4
7M-0	15	8	53	2	20
7M-1	1	0	0		
9M-0	14	3	21		
9M-1	9	2	22	1	33
9M-2	7	2	29		
10M-1	20	16	80		
10M-2	7	1	14		
14M-0	45	33	73	6	15
14M-1	24	17	71	3	15
15M-2	3	3	100		
18M-0	3	2	67	1	33
N=32				N_1=13	
Sum	363	204(56%)		26	
		Mean	52 (St.dev.35)		22 (St.dev.12)
		Median	55		20
		Range	0-100		4 -50

N, number of samples.
N1, number of samples with negative RT-PCR cell pellets.

The RT-PCR product could not be obtained from 26 mutants (13 pre- and 13 post-treatment) after testing at least four cell pellets per mutant. These mutants, found in 13 samples, comprised a mean of 22±12% (mean 20, range 4-50) of mutants in individual samples which were subjected to RT-PCR reaction (Table 4.6). The proportion of cDNA negative mutants was inversely proportional to the number of mutants analyzed by RT-PCR, but was slightly higher in samples from patient 5M (Table 4.6). The failure to produce a cDNA product in some mutants could result from a total deletion of the *hprt* gene or deletions in transcription regulatory part at the 5'-end of the gene. It is also possible that some cell pellets become unsuitable for the RT-PCR reaction as repeating the RT reaction on several cell pellets from mutants often gave positive results for some pellets though not for the others. Aging of the cell pellets also influenced the RT fidelity (data not shown).

The proportion of sequenced mutants comprised a mean of 52±35% of the selected mutants (Table 4.6). While the proportion of sequenced mutants varied, it was proportional to the number of selected mutants and a median of 55% of mutants selected from individual samples were sequenced (Table 4.6). Therefore, the obtained mutational spectrum adequately represents the spectrum in selected mutants.

Multiple mutations. After sequencing the mutants for the first time, 18 mutants (8 pre-melphalan and 10 post-melphalan) were found to contain one or more mutations or cDNA alterations additional to a base substitution (Table 4.7).

The RT-PCR reaction was then repeated on those mutants and the obtained cDNAs were sequenced. In the second round of RT-PCR the cDNA could not be obtained from 3

Table 4.7. List of pre- and post-melphalan mutants with complex mutations sequenced twice[a]. First round RT-PCR and sequencing.

MUTANT ID	MUTATION	POSITION	EXON	AA CHANGE	cDNA alteration	SEQUENCE
14m0-26	**TS G>A**, F-A	**173**, 265	3, 3	Gly>Glu	exon 2-3 loss in some	ATG G**G**A GGC, AAT **A**GT GAT
1m0-6	**TS G>A**, TS A>G	11, 611	1, 9	Arg>His, His>Arg		ACC C**G**C AGC, ttatagC**A**T GTT
1m0-27	**TS G>A**, TS G>A, SU	**358**, 539	4, 7	Asp>Asn, Asp>Asn	exon 8 loss	GGA **G**AT GAT, GTT G**G**A TTT
14m0-9	C TV A>C, D 8bp,	301, 304-311	3,3	Arg no change		ATC **A**GA **CTG AAG AG**C TAT
2m0-8	TV A>C, D10bp	622, 628-637	9, 9	Ile>Leu		GTC **A**TT AGT, AGT **GAA ACT GGA A**AA
14m0-36	TV C>G	368	4	Ser>stop	ex on 2-3 loss	CTC T**C**A ACT
14m1-8	SU, **TS G>A**	635	9	Gly>Glu	exon 2 loss	ACT G**G**A AAA
9m1-5	**TS C<T**, TS G>A	**235**, 419	3, 6	Leu no change, Gly>Asp		CTG **C**TG GAT, ACT G**G**C AAA
5m4-2	**TS G>A**, SU	**173**	3	Gly>Glu	exon 5 loss	ATG G**G**A GGC
10m1-13	TS G>A, SU	568	8	Gly>Arg	exon 2-3 loss in some	GTA **G**GA TAT
6m2-16	**TS G>A**, SU	**322**	4	Asp>Asn	17bp of exon 9 loss	AAT **G**AC CAG, **...ATT AG**T GAA
10m1-20	**TS G>A**, TS G>A	**165**, 212	3, 3	Lys no change, Gly>Asp		ATG AA**G** GAG, GGG G**G**C TAT
5m1-6	TS G>A, **TV A>C**	119, **502**	2, 7	Gly>Glu, Thr>Pro		CAT G**G**A CTA, AGG **A**CC CCA
10m1-19	SU, TV C>G	329	4	Ser>stop	exon 2-3 loss, exon 2-5 loss in some	CAG T**C**A ACA
5m1-3	C 16 TS G>A, SU	27,**119**,130,152,**159**, **238**,**268**,272,**285**,**292**, **302**,**306**,309,393,468, 569			17bp of exon 9 loss	

Table 4.7 (continuation). List of pre- and post-melphalan mutants with complex mutations sequenced twice[a]. Second round RT-PCR and sequencing.

MUTANT ID	MUTATION	POSITION	EXON	AA CHANGE	cDNA alteration	SEQUENCE
14m0-26	F-A	265	3		exon 2-3 loss in some	AAT ΔGT GAT
1m0-6	TS A>G	611	9	His>Arg		ttatagCΔT GTT
1m0-27	SU				exon 8 loss	
14m0-9	SU				exon 2-3 loss	
2m0-8	**TS G>A**, TV A>C, D10bp	**173**, 622, 628-637	3, 9, 9	Ile>Leu		ATG G**G**A GGC, GTC ΔTT AGT, AGT **GAA ACT GGA A**AA
14m0-36	TV C>G	368	4	Ser>stop		CTC T**C**A ACT
14m1-8	SU				exon 2 loss	
9m1-5	TS G>A	419	6	Gly>Asp		ACT G**G**C AAA
5m4-2	SU				exon 5 loss	
10m1-13	**TS G>A, TS G>A**, TS G>A	**173, 361**, 568	3, 4, 8	Gly>Glu, Asp>Asn, Gly>Arg		GAT **G**AT CTC, ATG G**G**A GGC, GTA **G**GA TAT
6m2-16	SU				17bp of exon 9 loss	...ATT AG**T** GAA
10m1-20	TS G>A	212	3	Gly>Asp		GGG G**G**C TAT
5m1-6	TS G>A	119	2	Gly>Glu		CAT G**G**A CTA
10m1-19	SU, TV C>G	329	4	Ser>UGA	exon 2-3 loss	CAG T**C**A ACA
5m1-3	C 28 TS G>A, SU	6,34,40,97,**119**, 135,139,154,**159**, 162,166,168,171, 172,173,208,229, **238,268,285,292**, **302,306**,322,337, 553,606,635			17bp of exon 9 loss	

[a] Mutants were sequenced twice using two cDNAs obtained from RT-PCR reactions on different cell pellets from each mutant. The mutations obtained in the second round sequencing are shown on the right side of the table.
Mutated bases are underlined, repeat and complement sequences are in bold, intron sequence is in small case. The position of mutations on cDNA is shown according to cDNA sequence with the A of the first transcribed codon being the first base (Edwards *et al.*, 1990). Mutations affecting intron sequence are shown according to the genomic sequence (Edwards *et al.*, 1990). TS- transition, TV-transversion, C- complex mutation, D-deletion, US- uncharacterized splice mutation, F-frameshift.
Base pair substitutions and their positions which were not confirmed after two round of RT-PCR and sequencing are in bold except for mutant 5m1-3 where mutated positions shared between the first and the second round cDNA sequence are in bold. Two bps at the sites of the unconfirmed mutated bases are in bold.

mutants (4m0-14, 14m0-4, 3m1-2), therefore the multiple mutations could not be confirmed in these cases.

Results for the rest of the mutants showed that the second round cDNAs of almost all of these mutants had only one mutation. There were three exceptions. The presence of both a transversion and a deletion was confirmed for mutant 2m0-8. However, an additional G>A transition appeared after the second round RT-PCR, which was not present in the first cDNA product (Table 4.7). The same was true for mutant 10m1-13 where two additional G>A transitions were detected in the product of the second round RT-PCR (Table 4.7). One of the transitions, position 173 of exon 3, might have been missed in the first round cDNA product because, contrary to the second product, it contained a mixture of two cDNA populations, a full length *hprt* cDNA and exons 2 and 3 lacking cDNA, which hampered the identification of exons 2 and 3 sequence in the first round cDNA product. The third mutant, 5m1-3, was interesting in that 16 GC>AT transitions were found scattered throughout the coding sequence after the first round RT-PCR and sequencing. The second round RT-PCR gave a cDNA product with 28 GC>AT transitions, eight of which were at the same positions as in the first sequence. Both of the cDNA products also had 17 bp missing from the beginning of exon 9, which was probably caused by a real mutation at the acceptor splice site of exon 9 in this mutant (Table 4.7).

The appearance of multiple base substitutions has been reported previously and was explained by misincorporations introduced by RT enzyme. Usually, another RT-PCR reaction on a different cell pellet from such mutants would rid the cDNA of multiple mutations (Curry *et al.*, 1997). The multiple mutations found in our mutants are also

likely due to RT misincorporations, although at least in three mutants we found even more mutations after the RT-PCR and sequencing were repeated with a new cell pellet from these mutants (Table 4.7). An interesting feature of the base substitutions not confirmed by the second round RT-PCR and sequencing is the prevalence of GC>AT transitions (10 out of 11 base substitutions) at 5'GA3' sequences (7 out of 10 base substitutions) (Table 4.7). Four of such base substitutions were found at position 173 (Table 4.7). All of the 16 multiple GC>AT transitions in mutant 5m1-3 in the first round RT-PCR occurred at GA sequences. In the second round RT-PCR product 24 transitions occurred at GA sequences, three tandem transitions occurred at GGGA sequence and the last transition occurred at the second base of GGGGGG repeat. The preferential occurrence of multiple GC>AT transitions at GA and GGGAA sequences has been reported previously (Curry *et al.*, 1997). The unusual case of multiple mutations in mutant 5m1-3 is probably caused by the interaction of the misincorporating activity of the RT enzyme and some factor(s) in this particular T-cell clone. These events are unlikely to be related to the melphalan exposure.

The mutation types. Of the 94 sequenced pre-treatment mutants no mutation altering the coding sequence was found in 7 mutants (Table 4.8). Mutations outside the coding sequence are probably responsible for the mutator phenotype in these mutants. It is also possible that mutation occurred at the splice sites of exon(s) which become spliced out but only in the proportion of the cDNA molecules (see below). Then, if the RT-PCR reaction preferentially amplifies the full-length cDNA, the splice mutation would not be detected. Two mutants had non-confirmed multiple mutations. Three samples (1M-0,

Table 4.8. List of sequenced *hprt* mutants selected from MM patients before melphalan/prednisone chemotherapy. Mutants were sorted first by the type of mutation and then by their position in the cDNA or by the type of cDNA alteration. Mutants were selected in the *hprt* T-cell clonal assay and the RT-PCR amplified mutant cDNAs were sequenced using ALF automated DNA sequencer. The exact sequence changes which resulted in aberrant splicing were determined in some cases by PCR-amplification and sequencing of the appropriate exon/intron region from the genomic DNA.

Mutant no.[a]	Mutation[b]	Position[c]	Exon	Amino acid change[d]	cDNA alteration[e]	Sequence[f]
nc 4m0-14	C TS T>C, TV A>T, TS G>A, TS T>C	27, 109, 173, 274	1, 2, 3, 3	Val>Ala, Ile>Phe, Gly>Glu, Ser>Pro		GTC GTGgtgag, TTT ATT CCT, ATG GGA GGC, AGA TCC ATT
nc 14m0-4	C TV T>G, TV C>G	407, 430	6,6	Ile>Arg, Gln>Glu		GAT ATA ATT, ATG CAG ACT
2m0-12	C D 8bp, IS A	11(12)-18(19), 10'	1, 1			ACC CGC AGC..CCT GGC
14m0-10	C SU				exon 8 loss, exon 2-3 loss	
14m0-9	C TV A>C, D 8bp, SU	301, 304-311	3,3	Arg no change	exon 2-3 loss in some	ATC AGA CTG AAG AGC TAT
2m0-8	C TV A>C, D10bp	622, 628-637	9, 9	Ile>Leu		GTC ATT AGT, AGT GAA ACT GGA AAA
1m0-10	D 15 bp	182-196	3			CAT CAC ATT...CTC TGT GTG
14m0-19	D 19bp	229-247	3			GCT GAC....ATC AAA
14m0-24	D 2bp	196-197(198-199 or 200-201)	3			GCC CTC TGT GTG CTC
6m0-1	SC D 34bp	26(27)-+32(33)	1 I1		IS 17(16)bp +33(34) (I1)-+49(I1)	GTC GTGgtg..cggt*tcagg..cag*gtg

14m0-35	D 3bp	162(163)-164(165)	3		GTG ATG AAG GAG
2m0-3	D 3bp	239(240)-241(242)	3		CTG GAT TAC
3m0-1	SC D 50bp	14(15-17)-+36 I1(37-40)	1 I1	IS 13(9)bp I1 +41-+49	CGC AGC CCT ...ttcaggcccacgcggcaggtg
14m0-20	D 8bp	511(512)-518(519)	8		CGA AGT..GGA TAT
14m0-26	F-A	265	3	exon 2-3 loss in some	AAT AGT GAT
3m0-2	F-T	532(533,534)	7 or 8		GAC Tgtaa..ttagTT
3m0-7	F +T	70'	2		TGC A'TA CCT
1m0-30	NF				
14m0-23	NF				
14m0-22	NF				
4m0-15	NF				
4m0-16	NF				
4m0-19	NF				
4m0-24	NF				
14m0-15	SU			17bp of exon 9 loss	...ATT AGT GAA
14m0-8	SU			21bp of exon 8 loss	...ATT CCA GAC
5m0-7	SU			exon 2-3 loss	
14m0-37	SU			exon 2-3 loss in some	
14m0-12	SU			exon 2-8 loss	
14m0-21	SU			exon 4 loss	
14m0-17	SU			exon 4 loss	
2m0-11	SU			exon 4 loss	
14m0-11	SU			exon 5 loss	
14m0-27	SU			exon 5 loss	
14m0-31	SU			exon 6 loss	

1m0-36	SU				exon 7 loss
1m0-07	SU				exon 7 loss
1m0-35	SU				exon 8 loss
1m0-27	SU				exon 8 loss
14m0-30	SU				exon 8 loss
3m0-4	SU				exon 8 loss
4m0-5	SU				exon 8 loss
7m0-12	SU				exon 8 loss
9m0-6	SU				exon 8 loss
14m0-29	SU				exon 8 loss in some
14m0-38	SU				exon 8 loss in some
7m0-2	T GA>TG	139-140	3		ACT GAA CGT
7m0-7	TS A>G	134	2	Arg>Gly	GAC AGgtaa
14m0-33	TS A>G	545	8	Glu>Gly	TTT GAA ATT
1m0-3[3]	TS A>G	611	9	His>Arg	ttatagCAT GTT
1m0-6[3]	TS A>G	611	9	His>Arg	ttatagCAT GTT
4m0-7[1]	TS A>G	611	9	His>Arg	ttatagCAT GTT
4m0-17[1]	TS A>G	611	9	His>Arg	ttatagCAT GTT
4m0-23[1]	TS A>G	611	9	His>Arg	ttatagCAT GTT
4m0-1[1]	TS A>G	611	9	His>Arg	ttatagCAT GTT
4m0-4[1]	TS A>G	611	9	His>Arg	ttatagCAT GTT
4m0-6[1]	TS A>G	611	9	His>Arg	ttatagCAT GTT
4m0-18[1]	TS A>G	611	9	His>Arg	ttatagCAT GTT
4m0-12[1]	TS A>G	611	9	His>Arg	ttatagCAT GTT
4m0-20[1]	TS A>G	611	9	His>Arg	ttatagCAT GTT
4m0-21[1]	TS A>G	611	9	His>Arg	ttatagCAT GTT
18m0-1[2]	TS C>T	74	2	Pro>Leu	ATA CCT AAT

18m0-2[2]	TS C>T	74	2	Pro>Leu		ATA C**C**T AAT
4m0-2	TS C>T	145	3	Leu>Phe		CGT **C**TT GCT
3m0-6	TS C>T	151	3	Arg>stop		GCT **C**GA GAT
7m0-4[h]	TS C>T	454	6	Gln>stop		AGG **C**AG TAT
14m0-6	TS C>T	508	7	Arg>stop		CCA **C**GA AGT
6m0-2	TS C>T	508	7	Arg>stop		CCA **C**GA AGT
9m0-9	TS G>A	3	1	Met>Ile		gttAT**G** GCG
1m0-4	TS G>A	197	3	Cys>Tyr		CTC T**G**T GTG
4m0-8	TS G>A	197	3	Cys>Tyr		CTC T**G**T GTG
14m0-16	TS G>A	212	3	Gly>Asp		GGG G**G**C TAT
7m0-13	TS G>A	568	8	Gly>Arg		GTA **G**GA TAT
9m0-7	TS G>A	569	8	Gly>Glu		GTA G**G**A TAT
3m0-8	TS G>A	617	9	Cys>Tyr		GTT T**G**T GTC
7m0-14	TS G>A	617	9	Cys>Tyr		GTT T**G**T GTC
1m0-03	TS T>C	110	2	Ile>Thr		TTT A**T**T CCT
1m0-5	TS T>C	437	6	Leu>Ser		ACT T**T**G CTT
14m0-39	TV C>A	222	3	Phe>Leu		AAA TT**C** TTT
5m0-1	TV C>G	222	3	Phe>Leu		AAA TT**C** TTT
14m0-36	TV C>G	368	4	Ser>stop	ex on 2-3 loss in some	CTC T**C**A ACT
7m0-15	TV C>G	464	6	Pro>Arg		AAT C**C**A AAG
5m0-4	TV C>G	610	9	His>Asp		ttatag**C**AT GTT
14m0-25	TV C>G	648	9	Tyr>stop		AAA TA**C** AAA
7m0-11[g]	TV G>C	119	2	Gly>Ala		CAT G**G**A CTA
14m0-40[g]	TV G>C	211	3	Gly>Arg	D 49(53) (exon 2)-570(574) (exon 8) in some	GGG **G**GC TAT, GGT **TAT GAC....GGA TAT GCC**
1m0-1	TV G>C	226	3	Ala>Pro		TTC TTT **G**CT GAC
14m0-42	TV T>A	2	1	Met>Lys		gttA**T**G GCG
14m0-34	TV T>A	595	8	Phe>Ile		TAC **T**TC AGG
1m0-2	TV T>G	104	2	Val>Gly		AGG G**T**G TTT

14m0-2	TV T>G	494	7	Val>Gly	CTG GTG AAA
14m0-5	TV T>G	542	8	Phe>Cys	GGA TTT GAA
5m0-3	TV T>G	596	8	Phe>Cys	TAC TTC AGG
2m0-6	TV T>G	612	9	His>Gln	ttatagCAT GTT

[a,b,c,d,e,f] Footnotes are the same as in Table 2.6.

[g] Mutants with GC>CG transversions at 5'GNC3' potential crosslink sites.

[h] Mutants with GC>AT transitions at 5'CpG3' sites.

[1,2,3] Mutants with an identical mutation obtained from the same sample.

Table 4.9 List of sequenced *hprt* mutants selected from MM patients after melphalan/prednisone chemotherapy. Mutants were sorted first by the type of mutation and then by their position in the cDNA or by the type of cDNA alteration. Mutants were selected in the *hprt* T-cell clonal assay and the RT-PCR amplified mutant cDNAs were sequenced using ALF automated DNA sequencer. The exact sequence changes which resulted in aberrant splicing were determined in some cases by PCR-amplification and sequencing of the appropriate exon/intron region from the genomic DNA.

Mutant no.[a]	Mutation[b]	Position[c]	Exon	Amino acid change[d]	cDNA alteration[e]	Sequence[f]
nc 3m1-2	C TS G>A, SU	173	3	Gly>Glu	exon 2 loss	ATG GGA GGC
nc 5m1-3	SU				17bp of exon 9 loss	
6m2-7	C D 6bp, IS +T	32-37, 31'	2, 2			ATT A'GT GAT GAT
4m2-3	C SU				exon 2-3, exon 6 loss	
10m1-19	C SU, TV C>G	329	4	Ser>stop	exon 2-3 loss, exon 2-5 loss in some	CAG TCA ACA
10m1-13	C TS G>A, TS G>A, SU	173, 568	3,8	Gly>Glu, Gly>Arg	exon 2-3 loss in some	ATG GGA GGC, GTA GGA TAT
3m1-1	D 26bp	157-182	3			GAT GTG ATG.....CAT CAC ATT
6m2-17	D 26bp	49-74	2			GGT TAT...ATA CCT AAT
10m1-4	D 38bp	212(214)-249(251)	3			GGG GGC TAT...AAA GCA
6m2-13	F+A	613'	9			CAT G'TT TGT
10m1-1	F+T	179(180)'				GGC CAT' T CAC
14m1-6	F-A	636(7,8,9)	9			ACT GGA AAA GCA
6m1-6	F-A	642(3,4,5)	9			GCA AAA TAC
5m1-4	F-G	171(172,173)	3			GAG ATG GGA GGC
6m1-8	F-T	223(4,5)	3			TTC TTT GCT
6m1-1	IS 38bp	28708-28745	I4			tcaggagc...ttaaggtat

5m1-9	NF				
5m3-2	NF				
6m2-22	NF				
6m2-25	NF				
6m2-5	NF				
6m2-1[1]	SC TS G>A	I5 +1	I5	IS 67bp of I5 +1-+67	GAA*gtaa...taaa*ggtaa
6m2-8[1]	SC TS G>A	I5 +1	I5	IS 67bp of I5+1-+67	GAA*gtaa...taaa*ggtaa
6m2-12[1]	SC TS G>A	I5 +1	I5	IS 67bp of I5+1-+67	GAA*gtaa...taaa*ggtaa
6m2-21[1]	SC TS G>A	I5 +1	I5	IS 67bp of I5+1-+67	GAA*gtaa...taaa*ggtaa
6m2-2[2]	SU			17bp of exon 9 loss	...ATT AGT GAA
6m2-10[2]	SU			17bp of exon 9 loss	...ATT AGT GAA
6m2-16[2]	SU			17bp of exon 9 loss	...ATT AGT GAA
10m1-2	SU			5bp of exon 2 loss	ttcagATT AGT GAT
5m1-10	SU			exon 2-3 loss	
5m4-6	SU			exon 2-3 loss	
5m4-10	SU			exon 2-3 loss	
5m4-9	SU			exon 2-3 loss	
14m1-14	SU			exon 2-3 loss	
4m2-8	SU			exon 2-3 loss	
5m4-5	SU			exon 2-3 loss, exon 5 loss in some	
5m4-11	SU			exon 2-3 loss in some	
6m2-11	SU			exon 2-3 loss in some	
10m1-21	SU			exon 2-4 loss	
14m1-12	SU			exon 2-6 loss	
6m2-20	SU			exon 2 loss	
14m1-8	SU			exon 2 loss	
9m1-2	SU			exon 3 loss	
1m1-1	SU			exon 4 loss	

14m1-16	SU				exon 4 loss	
6m2-14	SU				exon 4 loss	
10m1-12	SU				exon 4 loss	
10m1-14	SU				exon 4 loss	
5m1-11	SU				exon 5 loss	
5m4-2	SU				exon 5 loss	
4m2-6	SU				exon 5 loss	
5m2-16	SU				exon 6 loss, exon 2-3 loss in some	
14m1-11	SU				exon 8 loss	
9m2-1	SU				exon 8 loss	
6m1-3	SU				exon 8 loss in some	
6m2-4	SU				exon 8 loss in some	
6m2-24	SU				exon 8 loss in some	
6m1-4	T CC>GA	526, 527	7	Pro>Glu		AAG CCA GAC
14m1-29	TS A>G	499	7	Arg>Gly		AAA AGG ACC
4m2-1	TS A>G	611	9	His>Arg		ttatagCAT GTT
6m2-6	TS C>T	151	3	Arg>UGA		GCT CGA GAT
6m1-9	TS C>T	325	4	Gln>stop		GAC CAG TCA
14m1-1[3,b]	TS C>T	430	6	Gln>stop		ATG CAG ACT
14m1-17[3]	TS C>T	430	6	Gln>stop	47 (exon 2)-318 (end of exon 3) loss in some	ATG CAG ACT, CCA GGT...AAT GAC
14m1-19[3]	TS C>T	430	6	Gln>stop		ATG CAG ACT
14m1-2[3]	TS C>T	430	6	Gln>stop		ATG CAG ACT
14m1-3[3]	TS C>T	430	6	Gln>stop		ATG CAG ACT
14m1-4[3]	TS C>T	430	6	Gln>stop	exon 2-6 loss in some	ATG CAG ACT
6m1-5[h]	TS C>T	454	6	Gln>stop		AGG CAG TAT
5m1-6	TS G>A	119	2	Gly>Glu		CAT GGA CTA
1m1-3	TS G>A	134	2	Arg>Lys		GAC AGgtaag

14m1-15[4,h]	TS G>A	143	3	Arg>His		GAA C**G**T CTT
14m1-25[4]	TS G>A	143	3	Arg>His		GAA C**G**T CTT
6m2-23[h]	TS G>A	143	3	Arg>His		GAA C**G**T CTT
10m1-5	TS G>A	148	3	Ala>Thr		CTT **G**CT CGA
10m1-7	TS G>A	197	3	Cys>Tyr		CTC T**G**T GTG
10m1-22	TS G>A	208	3	Gly>Arg		AAG **G**GG GGC
5m4-7	TS G>A	209	3	Gly>Glu	exon 2-3 loss in some	AAG G**G**G GGC
10m1-20[5]	TS G>A	212	3	Gly>Asp		GGG G**G**C TAT
10m1-6[5]	TS G>A	212	3	Gly>Asp		GGG G**G**C TAT
5m2-7	TS G>A	212	3	Gly>Asp		GGG G**G**C TAT
9m1-5	TS G>A	419	6	Gly>Asp		ACT G**G**C AAA
15m2-2	TS G>A	539	8	Gly>Glu		GTT G**G**A TTT
9m2-5	TS G>A	569	8	Gly>Glu		GTA G**G**A TAT
6m1-7	TV A>C	498	7	Lys>Asn		GTG AA**A** AGG
15m2-3	TV A>T	109	2	Ile>Phe		TTT **A**TT CCT
5m2-5	TV A>T	643	9	Lys>stop		GCA **A**AA TAC
6m2-3	TV C>A	482	6	Ala>Glu		GTC G**C**A AG
10m1-8	TV C>G	74	2	Pro>Arg		ATA C**C**T AAT
5m2-1	TV C>G	113	2	Pro>Arg		ATT C**C**T CAT
6m1-2	TV C>G	113	2	Pro>Arg		ATT C**C**T CAT
5m1-5[g]	TV C>G	527	7	Pro>Arg		AAG C**C**A GAC
5m4-3[g]	TV C>G	527	7	Pro>Arg		AAG C**C**A GAC
5m2-9	TV G>C	118	2	Gly>Arg		CAT **G**GA CTA
6m2-19[g]	TV G>C	190	3	Ala>Pro		GTA **G**CC CTC
5m1-1[g]	TV G>C	211	3	Gly>Arg		GGG **G**GC TAT
6m2-18	TV G>C	601	8	Asp>His		AGG **G**AT TTG
14m1-7	TV G>T	97	2	Glu>stop		TTG **G**AA AGG
5m4-1[6,g]	TV G>T	229	3	Asp>Tyr		GCT **G**AC CTG
5m4-8[6]	TV G>T	229	3	Asp>Tyr		GCT **G**AC CTG

5m2-8[g]	TV G>T	580	8	Asp>Tyr	CTT GAC TAT
10m2-4	TV G>T	601	8	Asp>Tyr	AGG GAT TTG
6m2-9	TV G>T	601	8	Asp>Tyr	AGG GAT TTG
10m1-11	TV G>T	617	9	Cys>Phe	GTT TGT GTC
15m2-1	TV T>A	49	2	Tyr>Asn	GGT TAT GAC
10m1-17	TV T>A	104	2	Val>Glu	AGG GTG TTT
1m1-2	TV T>G	203	3	Leu>Arg	GTG CTC AAG
14m1-9	TV T>G	459	6	Tyr>stop	CAG TAT AAT
6m2-26	TV T>G	595	8	Phe>Val	TAC TTC AGG

[a,b,c,d,e,f] Footnotes are as in Table 2.6.

[g] Mutants with GC>CG transversions at 5'GNC3' potential crosslink sites.

[h] Mutants with GC>AT transitions at 5'CpG3' sites.

[1,2,3,4,5,6] Mutants with an identical mutation obtained from the same sample.

4M-0 and 18M-0) were found to contain more than one mutant with identical base substitution (Table 4.8). These mutants are probably clonal and only one mutant from each clone was included in the spectrum. A total of 11 clonal mutants were discarded from the final pre-treatment spectrum of 74 mutations (Table 4.8). Of the 109 sequenced post-treatment mutants, no mutation altering the coding sequence was identified in 5 cases (Table 4.9). Two mutants had non-confirmed multiple mutations while a total of 13 likely clonal identical mutants from six different clones were discarded from the final post-treatment spectra of 89 mutations (Table 4.9).

Base substitutions, insertions and splice mutants. All types of base substitutions were recovered and splice mutants resulting in the loss of one or several exons were present in both spectra (Tables 4.8 and 4.9). Most of the splice mutants were uncharacterized that means that the underlying mutation at the splice sites or exon sequence, which resulted in the aberrant splice event, was not determined. There were only three characterized splice mutations, two of which were deletions spanning the splice donor site of exon 1 resulting in an insertions of several base pairs of intron 1 into the cDNA (mutants 3m0-1 and 6m0-1) and one GC>AT transition at the splice donor site of exon 5 resulting in 67 bp insertion of intron 5 into the cDNA in several clonal mutants from sample 6M-2 (Tables 4.8 and 4.9).

Putative splice donor and acceptor sites were at the breakpoints of the inserted intron sequences or lost partial exon sequences in the characterized and uncharacterized splice mutants (Tables 4.8 and 4.9). The same was true for one insertion mutant, 6m1-1, where 38 bp from the middle of intron 4 was inserted into the cDNA sequence with AG and GT

putative splice acceptor and donor sites respectively at the breakpoints of the insertion (Table 4.9).

Deletions. A total of 11 deletion mutations were recovered, 8 from pre-melphalan and 3 from post-melphalan spectra ranging in size from 2 to 50 bp (Tables 4.8 and 4.9). Seven of the 11 mutants had deletions in exon 3 including two mutants from post-melphalan spectrum. Other deletions were scored in exons 2, 8 and at the exon/intron 1 junction. Direct repeats of 1 to 4 bp at the deletion breakpoints were found in 8 mutants indicative of polymerase slippage mechanism in their formation. Inverted complement sequences of 4 bp long were found in the other 2 mutants and the last mutant with deletion in exon 3 (mutant 14m0-19) had no repeats at the deletion breakpoints (Table 4.8). Deletions in exon 3 were clustered in a region with a potential for a secondary hair-loop structure formation, an association that has been reported previously (Pluth *et al.*, 1996).

Frameshifts and tandem mutations. All four -1 frameshift mutations in post-melphalan spectrum and two +T frameshifts in pre- and post-melphalan spectra each occurred at tandem repeats of the deleted or inserted bases suggesting the misalignment or slippage mechanism in their formation (Table 4.8 and 4.9). Three other frameshifts, -A in mutant 14m0-26, -T in mutant 3m0-2 and +A in mutant 6m2-13 occurred at sequences lacking tandem repeats.

There were two double tandem mutants. First, 7m0-2, had GA substituted with TG and the second, 6m1-4, had CC substituted with GA (Table 4.8 and 4.9).

Complex mutations. There were 4 pre-melphalan and 4 post-melphalan mutants demonstrating complex mutations (Table 4.8 and 4.9). Mutant 2m0-12 had 8 bp deletion in exon 1 and an insertion of an A just before the deletion. Similar events occurred in

mutant 6m2-7 where 6 bp deletion in exon 2 was accompanied by a +T insertion just before the deletion. In both cases neither repeats nor tandem sequences were found at the breakpoints of deletions or insertions. Two other pre-melphalan mutants had similar complex mutations. Mutants 14m0-9 and 2m0-8 both had an AT>CG transversion and a small deletion following the transversion several bp downstream. Mutant 14m0-9 also had exons 2-3 missing from some cDNA molecules. Two mutants had multiple exons missing from their cDNAs. Mutants 14m0-10 and 4m2-3 had exons 2-3, exon 8 loss and exons 2-3, exon 6 loss respectively. Two last complex mutants had base substitutions combined with splice mutations. Mutant 10m1-19 had a CG>GC transversion in exon 4 and exons 2-3 loss with exons 2-5 missing from some cDNAs. Mutant 10m1-13 had two GC>AT transitions in exons 3 and 8 combined with exons 2-3 loss in some cDNAs.

Loss of one or several exons in a proportion of cDNAs. Several mutants with one or several exons spliced out in the proportion of their cDNA were found (Tables 4.8 and 4.9). Three such mutants, 15m0-9, 10m1-13 and 10m1-19, were described in the previous section. Mutants 5m4-5 and 5m2-16 had exons 2-3 loss, exon 5 loss in some cDNAs and exon 6 loss, exons 2-3 loss in some cDNAs respectively. Four mutants (2 pre- and 2 post-melphalan) had alternative splicing resulting in exon loss in a proportion of cDNAs additional to a bp substitution or a frameshift which were either on the lost exon or outside it. Another 8 mutants (3 pre- and 5 post-melphalan) had either exon 8 or exons 2 and 3 loss from a proportion of their cDNA molecules (Tables 4.8 and 4.9). More interesting were two other mutants. Mutant 14m0-40 had a bp substitution in exon 3 and a large deletion found in a proportion of cDNAs that spanned several exons (from the middle of exon 2 to the sequence inside exon 8) flanked by 4 bp direct repeats. Mutant

14m1-17 had a bp substitution in exon 6 and a deletion from position 47 in exon 2 to an undetermined point downstream which resulted in the loss of exons 2 and 3 from some cDNA molecules. Mutants with an alternative cDNA splicing affecting some cDNA molecules are commonly found and have been described previously (Chapters II and III).

Spectra comparison. The post-treatment spectrum of 88 mutations was compared to the pre-treatment spectrum of 74 mutations and then compared to the spectrum of 337 mutations found in unexposed control population (Table 4.10). All three spectra appeared to be similar with noted differences being in the percentages of AT>GC transitions (4.5%, 8.1% and 2.2% in control, pre-melphalan and post-melphalan spectra respectively), GC>TA transversions (3.6%, 1.4% and 7.9%), AT>TA transversions (6.5%, 2.7% and 4.5%), -1 frameshifts (7.4%, 2.7% and 4.5%), deletions (8.6%, 8.1% and 3.4%) and complex mutations (2.7%, 5.4% and 4.5%). The only significant difference was in the percentage of GC>TA transversions, which increased in post-melphalan spectrum by almost six times compare to pre-melphalan spectrum (Table 4.10). The significance held true also when the spectra of only base pair changes were compared (Table 4.11).

Three out of seven GC >TA transversions in post-melphalan spectrum occurred at positions which have not yet been recovered in the control spectrum of 211 bp substitutions (positions 97, 229 and 482). Two GC>TA transversions from different samples occurred at position 601 which has been found mutated twice, and both times with this transversion in the control spectrum. Other two positions of GC>TA transversions in post-melphalan spectra (positions 580 and 617) were found mutated 3 (2

Table 4.10 Comparison of spectra of mutations found in unexposed control population (Curry *et al.*, submitted), and in multiple myeloma patients before and after melphalan/prednisone chemotherapy.

MUTATION CLASS	UNEXPOSED CONTROL		PRE-MELPHALAN		POST-MELPHALAN	
GC > AT	45	13.4%	14	18.9%	17	19.1%
AT > GC	**15**	**4.5%**	**6**	**8.1%**	**2**	**2.2%**
Transitions	*60*	*17.8%*	*20*	*27.0%*	*19*	*21.3%*
GC > TA [b]	**12**	**3.6%**	**1**	**1.4%**	**7**	**7.9%**
GC > CG	22	6.5%	8	10.8%	9	10.1%
AT > TA	**22**	**6.5%**	**2**	**2.7%**	**4**	**4.5%**
AT > CG	17	5.0%	5	6.8%	4	4.5%
Transversions	*73*	*21.7%*	*16*	*21.5%*	*24*	*27.0%*
TOTAL BP CHANGES	133	39.5%	36	48.7%	43	48.3%
Frameshifts - 1bp	**25**	**7.4%**	**2**	**2.7%**	**4**	**4.5%**
Frameshift + 1bp	5	1.5%	1	1.4%	2	2.2%
Splice errors [a]	129	38.3%	24	32.4%	31	35.0%
Deletions	**29**	**8.6%**	**6**	**8.1%**	**3**	**3.4%**
Complex	**9**	**2.7%**	**4**	**5.4%**	**4**	**4.5%**
Insertions	4	1.2%	0	0	1	1.1%
Tandem	3	0.9%	1	1.4%	1	1.1%
TOTAL	337	100%	74	100%	89	100%

[a] Splice errors include characterized and uncharacterized mutations which led to variant splice events.
[b] The proportion of GC>TA transversions is significantly higher in post-melphalan spectrum compared to pre-melphalan spectrum assuming expected equal distribution of mutations (χ^2=4.5, p<0.035).
Noted differences in spectra are in bold.

Table 4.11. Comparison of spectra of base pair substitution mutations in control unexposed population (Curry *et al.*, submitted) and in multiple myeloma patients before and after melphalan/prednisone chemotherapy.

MUTATION CLASS	UNEXPOSED CONTROL		PRE-MELPHALAN		POST-MELPHALAN	
GC > AT	45	33.8%	14	38.9%	17	39.5%
AT > GC	15	11.3%	6	16.7%	2	4.7%
Transitions	*60*	*45.1%*	*20*	*55.6%*	*19*	*44.2%*
GC > TA[a]	**12**	**9.0%**	**1**	**2.8%**	**7**	**16.3%**
GC > CG	22	16.5%	8	22.2%	9	20.9%
AT > TA	22	16.5%	2	5.5%	4	9.3%
AT > CG	17	12.8%	5	13.9%	4	9.3%
Transversions	*73*	*54.9%*	*16*	*44.4%*	*24*	*55.8%*
TOTAL BP CHANGES	133	100%	36	100%	43	100%

[a] The proportion of GC>TA transversions is significantly higher in post-melphalan spectra compare to pre-melphalan spectra (χ^2=4.5, $p < 0.035$).

Table 4.12. Distribution of mutations affecting exon splicing in the *hprt* gene in control unexposed population and multiple myeloma patients before and after melphalan/prednisone chemotherapy.

Exon	Unexposed control		Pre-melphalan		Post-melphalan	
	N	(%)	N	(%)	N	(%)
1	1	1	2	8	0	0
2[a]	38	29	3	13	14	45
3	3	2	0	0	1	3
4	18	14	3	13	5	16
5	9	7	2	8	4	13
6	10	8	1	4	1	3
7	9	7	2	8	0	0
8	33	26	10	42	5	16
9	8	6	1	4	1	3
Total splice	129	100	24	100	31	100

[a] Includes splice mutants with exon 2 loss as well as mutants with losses of multiple exons beginning with exon 2.

GC>AT and 1 GC>TA) and 8 (5 GC>AT and 3 GC>TA) times respectively in the control spectrum. The other noted differences in the spectra may be due to relatively small sample sizes in pre-and post-melphalan spectra compare to a much larger sample size for the control spectra. Some differences appear in the distribution of mutations that affected the exon splicing (Table 4.12). Exons 2 (2 and 3), 8 and 4 were affected the most in all spectra, however, exon 8 was more affected in the pre-melphalan spectra and exon 2 (2 and 3) were more affected in post-melphalan spectrum when three spectra were compared. The significance of these differences is unclear, as the clonality of splice mutants from the same sample with identical cDNA changes can not be excluded. An increase in the proportion of mutants affecting splicing of exon 1 was noted in the pre-melphalan spectrum (Table 4.11).

There was no significant difference in the distribution of deletion mutations although the number of deletion mutations recovered from the patients was significantly lower than that scored in the control spectrum (Table 4.10 and 4.13). Most of the deletions recovered from the patients were in exon 3 (63% in pre-melphalan and 67% in post-melphalan spectra) (Table 4.13). Exon 3 together with exon 2 both contain the highest number of deletion mutations in the spectrum from the control unexposed population (24% and 30% of deletion mutations respectively) (Table 4.13).

There was no difference among the three spectra in the distribution of bp substitutions with exon 3 having the largest number of bp substitutions followed by exons 8 and 2 (Table 4.14). A slight increase in the proportion of bp substitutions was found in exon 9 in pre-melphalan spectrum and in exon 2 in post-melphalan spectra.

Table 4.13 Distribution of deletion mutations including characterized splice mutations in spectra from control unexposed population and MM patients before and after melphalan/prednisone chemotherapy.

Exon[a]	Control unexposed		Pre-melphalan		Post-melphalan	
	No.	%	No.	%	No.	%
1						
2	10	30			1	34
3	8	24	5	63	2	67
4	2	6				
5						
6	4	12				
7	1	3				
8	3	9	1	13		
9	1	3				
I1	1	3	2	25		
I2	2	6				
I7	1	3				
Total	33	100%	8	100%	3	100%
Size	2-66 bp		2-50 bp		26-38 bp	

[a]I, intron. Deletions of the intron/exon junction sequences leading to splice mutations.

Table 4.14. Distribution of base pair substitutions in the control, pre-melphalan and post-melphalan spectra.

Exon	Control		Pre-melphalan		Post-melphalan	
	N	(%)	N	(%)	N	(%)
1	12	6	2	6	0	0
2	36	17	5	14	10	23
3	57	27	9	25	13	30
4	1	0.5	1	3	1	2
5	5	2	0	0	0	0
6	17	8	3	8	5	12
7	22	10	3	8	4	9
8	41	19	6	17	7	16
9	20	10	7	19	3	7
Total	211	100	36	100	43	100

Table 4.15. List of sequenced *hprt* mutants from pre- and post-melphalan samples from patient 5M.

Mutant no.[a]	Mutation[b]	Position[c]	Exon	Amino acid change[d]	cDNA alteration[e]	Sequence[f]
5M-0 (7)[i]						
5m0-3	TV T>G	596	8	Phe>Cys		TAC TTC AGG
5m0-1	TV C>G	222	3	Phe>Leu		AAA TTC TTT
5m0-4	TV C>G	610	9	His>Asp		ttatagCAT GTT
5m0-7	SU				exon 2-3 loss	
5M-1 (16)[i]						
5m1-5[g]	TV C>G	527	7	Pro>Arg		AAG CCA GAC
5m1-10	SU				exon 2-3 loss	
5m1-9	NF					
5m1-1[g]	TV G>C	211	3	Gly>Arg		GGG GGC TAT
5m1-11	SU				exon 5 loss	
nc 5m1-3 (1)	C 16 TS G>A, SU	27,119,130,152,159, 238,268,272,285,292, 302,306,309,393,468, 569			17bp of exon 9 loss	
nc 5m1-3(2)	C 28 TS G>A, SU	6,34,40,97,119,135,139, 154,159,162,166,168,17 1,172,173,208,229,238, 268,285,292,302,306,32 2,337,553,606,635			17bp of exon 9 loss	
5m1-6	TS G>A	119	2	Gly>Glu		CAT GGA CTA
5m1-4	F -G	171(172,173)	3			GAG ATG **GG**A GGC
5M-2 (17)[i]						
5m2-1	TV C>G	113	2	Pro>Arg		ATT CCT CAT
5m2-5	TV A>T	643	9	Lys>stop		GCA AAA TAC

5m2-8[g]	TV G>T	580	8	Asp>Tyr		CTT GAC TAT
5m2-16	SU				exon 6 loss, exon 2-3 loss in some	
5m2-9	TV G>C	118	2	Gly>Arg		CAT GGA CTA
5m2-7	TS G>A	212	3	Gly>Asp		GGG GGC TAT
5M-3 (8)[i]						
5m3-2	NF					
5M-4 (12)[i]						
5m4-6	SU				exon 2-3 loss	
5m4-10	SU				exon 2-3 loss	
5m4-7	TS G>A	209	3	Gly>Glu	exon 2-3 loss in some	AAG GGG GGC
5m4-9	SU				exon 2-3 loss	
5m4-2	SU				exon 5 loss	
5m4-3[g]	TV C>G	527	7	Pro>Arg		AAG CCA GAC
5m4-8[6,g]	TV G>T	229	3	Asp>Tyr		GCT GAC CTG
5m4-11	SU				exon 2-3 loss in some	
5m4-5	SU				ex 2-3 loss, exon 5 loss in some	
5m4-1[6,g]	TV G>T	229	3	Asp>Tyr		GCT GAC CTG

[a-g] Footnotes as in Table 4.8.

[6] Footnote as in Table 4.9.

[i] The number in brackets is the number of selected mutants in the individual samples.

Mutations found in the first (5m1-3(1)) and the second (5m1-3(2)) round RT-PCR and sequencing are shown for mutant 5m1-3.

Patient 5M. To try to explain a significant increase in post-treatment MF in patient 5M mutants obtained from this patient were investigated more closely (Table 4.15). Five pre-treatment mutants were four transversions and one exons 2 and 3 splice mutant. The post-treatment mutants were 9 transversions, 9 splice mutants, 3 GC>AT transitions, one frameshift, 2 mutants with no found mutation and one mutant, 5m1-3, with not confirmed multiple GC>AT transitions and a splice mutation. There were three GC>TA transversions among the post-treatment mutants, however, two of them (mutants 5m4-1 and 5m4-8) are at the same position and from the same sample and are probably clonal. The proportion of GC>TA transversions was significantly increased in the overall post-melphalan spectrum compared to pre-treatment spectrum in this study and this change could have been induced by melphalan (see below). Unfortunately, it was possible to obtain the sequence of only one out of 8 selected mutants from sample 5m-3 which had a significant elevation in MF (Table 4.15). This mutant had no cDNA mutation. Almost all (10 out of 12) selected mutants were sequenced from sample 5m-4 that also had elevated MF. An interesting feature of these mutants is that 4 of them had exons 2 and 3 loss and the other two had exons 2 and 3 missing in a proportion of the cDNA molecules. Although the splicing out of the exons could be due to various mutations at the splice sites, the possibility that some of these mutants are clonal is not excluded. The proportion of exon 2 and 3 loss mutants was increased in the overall post-melphalan spectrum (Table 4.12).

5. Discussion.

***hprt* MF.** This study of nine multiple myeloma patients showed that melphalan/prednisone chemotherapy did not significantly increase mutation in the

majority of the patients. This result is similar to another study of 12 connective disease patients treated with chlorambucil, bifunctional alkylating agent similar to melphalan, and to our previous study of etoposide chemotherapy in small cell lung cancer patients where no elevation in the *hprt* MF was observed after treatment (Karnaoukhova *et al.*, 1997, Palmer *et al.*, 1986). There was also no significant elevation in the *hprt* MF in breast cancer and germ cell tumor patients after combination chemotherapy (Perera, *et al.*, 1992, Sala-Trepat *et al.*, 1990). This, however, contrasts with several studies where a significant increase in the *hprt* MF was observed in patients after treatment. Bifunctional alkylating agent, cyclophosphamide, as a single agent or given in combination with other cytotoxic drugs has been associated with mutation induction in all studies (Ammenheuser *et al.*, 1988, Palmer *et al.*, 1988, Branda *et al.*, 1991). Other alkylating agents used in combination or together with non-alkylating drugs have also been associated with an increased *hprt* MF after treatment (Tates *et al.*, 1994, Caggana *et al.*, 1991). Several reasons can be suggested to explain the differences seen in these studies. It is possible that only some specific agents or their combination can induce significant elevations in the *hprt* MF. Other factors such as experimental variation, expression and persistence of mutations and the complex biology of T cells can also influence the results. Also, the variation in MF determination can also contribute to the negative results. We observed up to 3-fold variation in MF when the assay was performed on split samples (Table 4.4), while others have found that the MF for any individual donor may vary considerably between different samples from a single donor (2-8-fold) (Cole *et al.*, 1994).

Timing might be important. Using an autoradiographic assay, Ammenheuser *et al.* (1988) have observed that the MF reached its maximum at 2 weeks post-treatment and

then declined to pre-treatment levels by 4 weeks. If that were the case in this melphalan study, we would have not be able to detect the transient elevations in MF since the blood samples in our study were usually taken on the day the treatment cycles began or after almost a month since the end of the previous cycle (Table 4.3). However, persistently elevated MF have been detected in cancer patients months and years after the completion of chemotherapy (Tates *et al.*, 1994, Branda *et al.*, 1991, Caggana *et al.*, 1991). In fact, the expression time for the induced mutations in the *hprt* gene after combination chemotherapy has been determined to be 98±54 days in one study (Tates *et al.*, 1994). Interestingly, samples 5M-3 and 5M-4 where the significantly elevated MF values were detected in patient 5M were taken 3.8 and 5.7 months after the last previous melphalan/prednisone cycle which is the longest post-treatment time prior to the blood sample in this study (Table 4.3).

As in the other studies we observed an interpatient variability in MF changes with some patients having higher MF values after treatment compared to the pre-treatment values. In particular, one patient, 5M, had a steady increase in MF over the treatment period (Figure 4.1). The MF in this patient became significantly elevated compared to pre-treatment value after 15.1 months and a total dose of 728 mg of melphalan and increased even more 8 months later after two additional cycles of melphalan and three cycles of dexamethasone (Figure 4.1). It is possible, however, that near 7-fold increase in MF in samples 5M-3 and 5M-4 was confounded by a drop in CE in these samples compared to the previous samples from this patient (Table 4.4, Figure 4.1) as lnMF is inversely proportional to CE. It is interesting to note that patient 5M received the highest

melphalan dose and this patient was followed for the longest time compared to the other patients (Figure 4.1). It is possible that melphalan chemotherapy has a cumulative effect on the mutation induction and that the significant increase in the *hprt* MF can be detected only after a certain duration of the therapy and a sufficient accumulation of long-lived mutant T-lymphocytes in the peripheral blood. This would explain the elevated MF in patient 5M and the absence of such elevation in other patients. Consistent with this assumption is the fact that the risk of secondary malignancy is dose-dependent and patients receiving higher dose have a higher risk of developing a secondary cancer (Levine *et al.*, 1992). Positive correlation between the *hprt* MF and the duration of cancer chemotherapy in patients has been observed previously (Palmer *et al.*, 1988). It is also possible that cells in patient 5M became adapted to the cytotoxic chemotherapy treatment over time. Some populations of T cells might have become less sensitive to apoptosis and, therefore, more mutated cells could be recovered. Interestingly, the MF did not change much from sample 5M-3 to sample 5M-4 taken almost 8 months later and after additional 2 cycles of melphalan and three cycles of dexamethasone indicating that the later glucocorticoid treatment was not mutagenic.

It is also possible that the low CE values obtained in our experiments confounded the results causing the pre-treatment MF values to be relatively high which might have smoothed the possible mutagenic effect. The pre-treatment MF for this group of MM patients (mean MF, $47\pm37\times10^{-6}$) is much higher than the MF for the age-matched unexposed control population (mean MF, 15.1±9.5) (Curry *et al.*, submitted). The high MF values could in part be attributed to relatively low CE in our samples compared to the

CE in the control population (10±5 *vs.* 38.4±18.6 respectively), as lnMF is inversely proportional to CE (Table 4.5). The factors that could have influenced the CE and MF in our samples are the patients smoking history (8 out of 9 patients admitted to being current or ex-smokers) and the disease state (Table 4.1). Smoking has been associated with an increase in *hprt* MF (Cole *et al.*, 1994) and lower CE and higher MF values in cancer patients before treatment compared to age-matched healthy unexposed controls have been reported in some studies (Caggana *et al.*, 1991, Karnaoukhova *et al.*, 1997).

Apart from obtaining overall lower CE and higher MF values, we observed similar lnMF=f(age, CE) relationships with those for the control unexposed population and those obtained in our previous etoposide study. In 55-85 years age group we observed that lnMF and CE are independent of age and that the lnMF is inversely proportional to CE. Although insignificant for samples in the melphalan study, which is probably related to the small sample size, the later relationship became significant when both etoposide and melphalan samples were considered together (Table 4.5).

***hprt* mutational spectra.** The spectrum of the *hprt* mutations in patients after melphalan treatment was found to be similar to the pre-treatment spectrum from these patients, however, one significant difference was in the proportion of GC>TA transversions which were increased in the post-treatment spectrum compared to pre-treatment spectrum (Table 4.10). The proportion of GC>TA transversions was not significantly different, however, in the post-melphalan spectrum compared to that from the control, unexposed population. Other differences between pre- and post-melphalan spectra such as decrease with treatment in the proportion of AT>GA transitions and deletions were not significant. The

pre-melphalan spectrum, on the other hand, was found similar to the spectra of mutation in the control unexposed population. There were no significant changes between the two spectra, however, several differences were noted such as twice the proportion of AT>GC transitions and complex mutations and nearly 2.5 times decrease in the proportions of GC>TA, AT>TA transversions and -1 frameshifts in patients compared to the control population (Table 4.10). Such differences may simply reflect the smaller sample size in pre-melphalan spectrum compared to the control spectrum.

An increase in MF in patient 5M can be due to treatment or be a consequence of clonal proliferation of *hprt* deficient mutants in the post-treatment samples. The spectrum of sequenced mutants from this patient, though not big and complete enough to make definite conclusions suggests that both of these possibilities could have played a role (Table 4.15).

Melphalan is expected to produce mutations at both GC and AT base pairs (Povirk *et al.*, 1994). Such mutations would result from DNA damage melphalan known to induce: monoalkylation adducts at N7-guanine and N3-adenine, G-G or A-G intrastrand crosslinks and interstrand crosslinks between two guanines at 5'GNC" sequences (Povirk *et al.*, 1994). Consistent with this model, melphalan was reported to induce base substitutions at both AT and GC base pairs but in different systems (Wang *et al.*, 1990, Austin *et al.*, 1992). The significant increase in GC>TA transversions in our post-melphalan spectrum is consistent with the mutational spectra of melphalan in the CHO *aprt* locus (Austin *et al.*, 1992). In this system mutations at GC base pairs, especially GC>TA and GC>CG transversions, predominated and were found to cluster at 5'GGC3' and 5'GGCC3' potential crosslink sites (Austin *et al.*, 1992). Seven GC>TA

transversions in our post-melphalan spectrum were recovered from different patients. Patients 5m, 6m and 10m each had two GC>TA transversions and patient 14m had one GC>TA transversion. Interestingly, 4 out of 9 GC>CG transversions (3 of them from patient 5m) and 2 out of 7 GC>TA transversions (both from patient 5m) in the post-melphalan spectrum occurred at 5'GNC3' potential interstrand crosslink sites, while only 2 out of 8 GC>CG transversions and not the one GC>TA transversion in the pre-melphalan spectra occurred at those sites (Tables 4.8 and 4.9).

Although no elevation in mean MF was detected after melphalan treatment, post-treatment spectrum changes suggest that the mutational spectrum might be a more informative marker of the mutagenic exposure than the MF in cancer patients after chemotherapy.

6. Conclusion.

The carcinogenic potency of alkylating agent chemotherapy is well documented (Povirk *et al.*, 1994, Bergsagel *et al.*, 1979, Curtis *et al.*, 1984, Levine *et al.*, 1992, Gori *et al.*, 1983). Approximately 4% of multiple myeloma patients treated with alkylating agent chemotherapy including melphalan develop leukemia with the actuarial risk reaching 17% at 4 years (Bergsagel *et al.*, 1979, Rodjer *et al.*, 1990, Hussein 1994). Cytogenetic abnormalities in sAML complicating melphalan-treated multiple myeloma were found to be very specific with -5/5q- and/or -7/7q- abnormalities being the most common (Mamuris *et al.*, 1990). As possible explanation for the leukemogenic potential of alkylating agents it has been proposed that treatments with alkylating agents induce nonrandom mutations of recessive genes located on the chromosomes affected by the rearrangement in sAML. Various rearrangements then occur including deletions of the

homologous normal counterparts, unmasking mutated recessive genes (Povirk *et al.*, 1994, Mamuris *et al.*, 1990). Although the candidate tumor suppressor genes have been identified at the 5q31 and 7q31 chromosome bands, which are most frequently deleted in sAML cases, it is still unknown what kinds of mutations are primarily responsible for the inactivation of these genes leading to sAML (Pedersen *et al.*, 1994, Le Beau *et al.*, 1993).

Our study demonstrated that mutations are not readily induced in lymphocytes of multiple myeloma patients after melphalan chemotherapy. However, the prolonged exposure (over a year) to high melphalan doses (728-840 mg) was associated with the mutation induction in one patient and might be mutagenic. The increase of GC>TA transversions in post-melphalan spectra is consistent with the melphalan-induced DNA adducts and cross-links at guanines and might be treatment-related. The study of more patients with the prolonged exposure to high doses of melphalan is needed to provide additional support for these observations.

The identification of the types of mutations in cancer genes responsible for the sAML following chemotherapy with alkylating agents will provide an understanding of the carcinogenic specificity of these agents. Until then, it is hard to predict what types of mutations that might be induced by melphalan chemotherapy in peripheral blood lymphocytes of cancer patients can translate in the lesions in cancer genes and lead to the development of secondary leukemia.

CHAPTER V. FISH ANALYSIS OF THE *MLL* GENE CHROMOSOMAL REARRANGEMENTS IN SMALL CELL LUNG CANCER (SCLC) PATIENTS RECEIVING ETOPOSIDE CHEMOTHERAPY

1. Abstract.

Rearrangements of the MLL gene on chromosome band 11q23 are found in >50% of secondary acute myeloid leukemias (sAML) in cancer patients previously treated with epipodophyllotoxins. This suggests the high degree of susceptibility of this chromosomal region to chemotherapy drugs targeting topoisomerase II. Most of the MLL gene rearrangements involve reciprocal translocations of the 5' end of the MLL gene to the 3' end of one of the partner genes. Using fluorescence *in situ* hybridization (FISH) with genomic DNA probe spanning a breakpoint cluster region (brc) in the MLL gene we monitored a group of SCLC patients receiving etoposide chemotherapy for the appearance of MLL gene rearrangements in patients' unstimulated peripheral blood mononuclear cells. Results from pre-treatment patient's samples determined the cut-off level for the detection of MLL gene rearrangements being 4.4% or 1 rearranged cell in 23 normal cells. There was no increase in the number of rearranged cells in patients after treatment. This result is not surprising since the sensitivity of the FISH assay (1 rearranged cell in 44 normal cells) is much lower than is required for the detection of presumably rare events of MLL gene rearrangement induced in peripheral blood cells of SCLC patients by etoposide chemotherapy. While FISH is useful in detecting MLL rearrangements in leukemia patients, it is suggested that more sensitive technique such as

RT-PCR should be used for monitoring cancer patients receiving epipodophyllotoxin chemotherapy for the appearance of MLL gene rearrangements.

2. Introduction.

Identification and characterization of *MLL* gene. Leukemia-associated reciprocal translocations of 11q23 chromosome band occur with high frequency in several well-defined clinical settings. Chromosomal translocations in this region occur in 70% of monoblastic variants of AML in infants and young children, in 60% of ALL in the first year of life (compared to 6% of ALL and AML in older children and adults), and in over 50% of epipodophyllotoxin-related leukemias (Felix *et al.* 1995 (1,2)).

Pulsed-field gel electrophoresis (PFGE) and FISH have both been used to map the region containing the 11q23 breakpoints in leukemia (Rowley, 1993). FISH identified that the breakpoint lies telomeric to the CD3G gene and centromeric to the PBGD gene (Figure 5.1). An yeast artificial chromosome (YACyB22B2) that contained a 330 kb insert of human DNA with the CD3 gene cluster at the centromeric end of the YAC was shown to span the breakpoint region in most leukemias with 11q23 rearrangements. Subsequently, a gene named *MLL* for myeloid-lymphoid leukemia or mixed-lineage leukemia that spans the breakpoint in these translocations was identified. The *MLL* gene is 100 kb long and contains at least 21 exons. It encodes a large protein of 3,968 amino acids that has several regions homologous to the product of the *Drosophila* developmental gene *trithorax (trx)* (Figure 5.1). The TRX protein is involved in the transcriptional maintenance of genes of homeotic type during *Drosophila* embryogenesis. The product of *MLL* exhibits a region similar to the AT hook of high-mobility -group protein. This protein binds to AT-rich regions of the minor groove of the DNA double

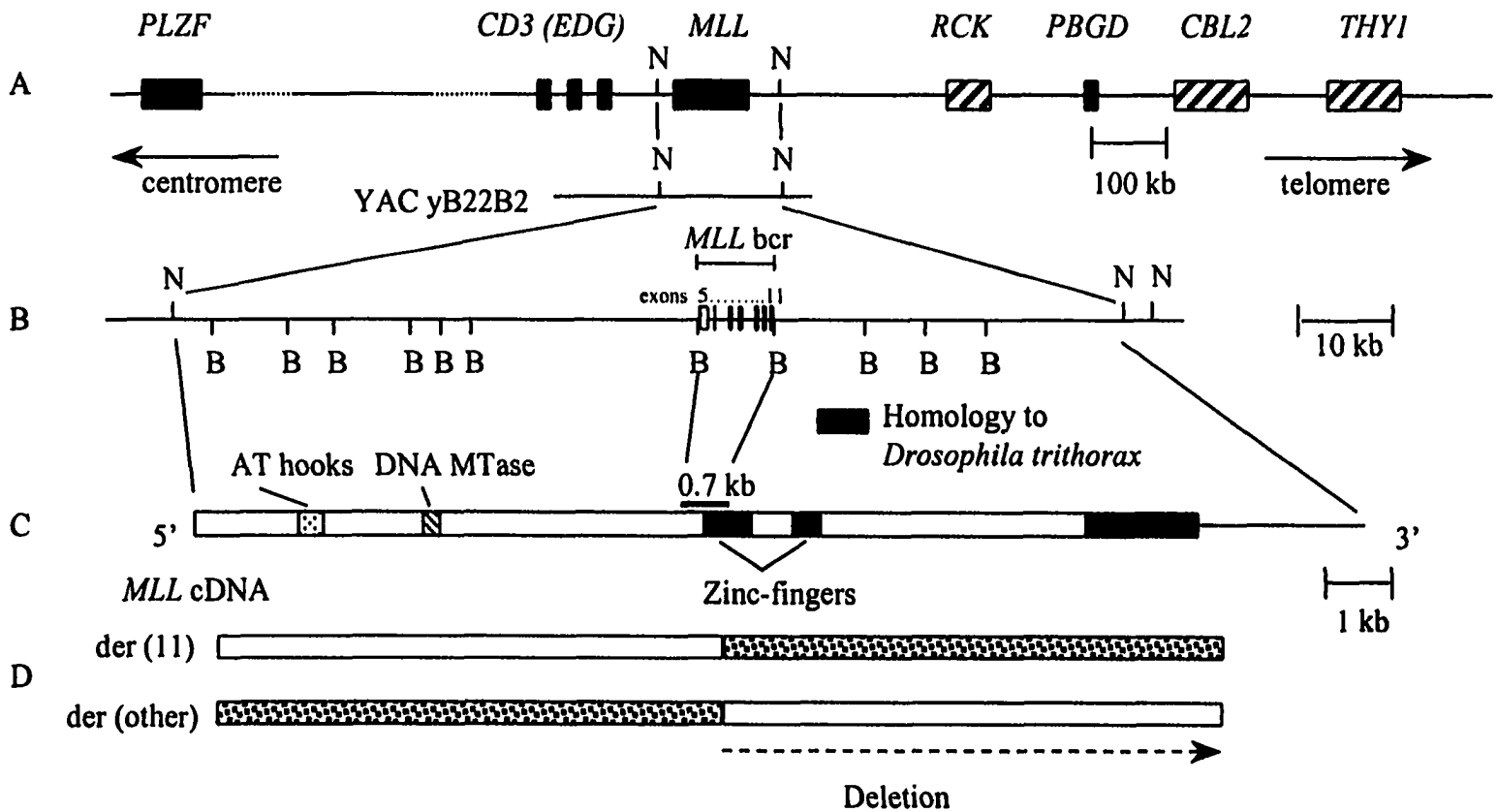


Figure 5.1 (A) A partial map of 11q23 chromosome band showing the location of nearby genes. The positions not precisely mapped genes RCK, CBL2 and THY1, are shown by hatched boxes. Below, partial restriction map of YAC showing alignment to larger genomic map above and to the MLL gene in B. (B) A partial map of the MLL gene showing the location of the 8.3 kb breakpoint cluster region (bcr) and schematic location of exons 5 to 11. (C) Diagram of the MLL cDNA with the position of the 0.74 kb probe and the general location of important motifs. (D) Representation of two derivative chromosomes formed as a result of translocation. The zinc-fingers are translocated to the der (other) chromosome and part or all of MLL telomeric to the breakpoint is deleted in about 25% of *de novo* acute leukemia patients. N, NotI; B, BamHI; der(11), derivative 11 chromosome; der(other), other derivative chromosome. (Adapted from Rowley, 1993.)

helix. It cannot directly activate transcription, but it facilitates the action of other factors such as NF-κB. The MLL protein also possesses a cystein-rich region (CRR) homologous to mammalian DNA methyltransferases. In these proteins, the CRR binds zinc and could be involved in discriminating between hemimethylated and unmethylated DNA. This is relevant, as the state of methylation of the target gene may influence the ability of MLL to regulate its expression. The MLL protein also exhibits cysteine- and histidine-rich regions homologous to the TRX protein that could form a particular type of zinc finger (6-10 zinc fingers) and could constitute a DNA binding domain (Bernard *et al.*, 1995). The zinc finger motifs are similar to those of the steroid binding hormones. These data suggests that the human *MLL* gene is a transcriptional regulation factor that possesses at least two DNA binding domains. The *MLL* gene is transcribed from centromere to telomere and is expressed in a wide variety of human tissues, including spleen, liver, brain and in both T and B cell lines.

***MLL* rearrangements and partner genes.** Abnormalities of 11q23 involving MLL gene in leukemias mostly result in fusion transcripts between *MLL* and the partner gene located on the chromosome involved in the reciprocal translocation (Bernard *et al.*, 1995). These fused transcripts can code for chimeric proteins. Almost all of the breakpoints characterized are clustered within a 8.3 kb region located between exons 5 and 11 of the gene (Figure 5.1). No difference in the *MLL* breakpoint location has been found between *de novo* and secondary leukemias in the patients studied. Also, the localization of breakpoints does not seem to be related to the cellular type of malignant proliferation. The *MLL* exons 6-10 are small (74-147 bp) and they end at the third base of the codon.

This results in the same open reading frame from the splicing of each one. Indeed, the chimeric RNA fusion points are situated at the splicing sites of exon 6, 7, or 8 depending on the position of the breakpoint.

Around 40 different chromosomal bands have been described which are involved in rearrangements with 11q23 in leukemia with 27 of them being rearranged with *MLL* gene (Berenard *et al.*, 1995, Rowley 1993, Canaani *et al.*, 1995). Two fusion genes and consequently two chimeric proteins potentially result from such translocations. One is located on the derivative chromosome 11 (der(11)) and codes for the NH2 part of *MLL* fused with the COOH part of the partner. The other lies on the derivative partner chromosome (der(other)) and encodes the NH2 side of the gene fused with the COOH side of *MLL*. The importance of the der(11) chromosome in leukemogenesis is supported by three arguments: in variant complex translocations involving band 11q23, the junction corresponding to der(11) that bears the exons encoding the NH2 side of *MLL* is formed, whereas the distal part of 11q which bears the *MLL* exons encoding the COOH part of the gene, is translocated onto another chromosome; the *MLL* sequences that are telomeric to the transcription breakpoint (COOH side) are deleted in some 20-25% of patients; transcription studies have shown that the 5'-partner-*MLL*-3' fusion messengers were inconsistently expressed, whereas the reciprocal messenger was well expressed (Bernard *et al.*, 1995).

The chimeric proteins can alter the normal function of the MLL protein in several ways. The fusion gene retains the A-T hook and MT domains but lacks the zinc fingers of the MLL. Therefore, the fusion proteins are probably losing the specific DNA -binding of

MLL due to abolished zinc-fingers, however, may retain a nonspecific DNA binding ability and can modify the methylation status of DNA and therefore affect transcription.

Eight partner genes have been identified (Table 5.1) (Bernard *et al.*, 1995). Some of them such as AF10 and AF17, and ENL and AF9 are closely related at the structural and functional level and code for nuclear transcription factors. The chimeric protein can neutralize the normal function of MLL by competing for its normal targets and/or by sequestering the normal MLL protein. It can also constitute a new transcription factor by fusion of a DNA binding motif (AT hook) with a currently unidentified trans- activation domain in the product of the partner gene. Others, like AF1p and AF6, encode cytoplasmic proteins and might act through their dimerization domains to form homodimers or heterodimers which could efficiently occupy MLL DNA target sites and prevent transcription of these targets (Canaani *et al.*, 1995).

With the exception of AF10 and AF17 and of ENL and AF9, it now appears clear that the partner genes of MLL are not closely related at the structural or functional levels. The variety of MLL partners in 11q23 rearrangements suggests that the partners could have only a minor role and could be interchanged in leukemogenesis. On the other hand, the predominant phenotype of the leukemic cells is determined by the genes contributed by the partner chromosome (Rowley, 1993). In ALL cases, the t(4;11) is found in half of the cases, whereas the t(11;19) occur less frequently; in AML cases, the t(9;11) predominates, and the t(11;19) and t(6;11) are less common (Martinez-Climent *et al.*, 1995). The explanation for these associations could be due either to the nature of the partner genes or to the recombination mechanisms, such that a given gene could be rearranged at certain steps of hematopoietic differentiation. Infants with lymphoblastic

Table 5.1 Characterized partners of MLL gene in leukemia 11q23 translocations.

Position	Gene	Protein type or structural domains	Disease
1p32	AF1P	eps15 homologue, cytoplasmic	AML-M5, -M0, ALL
4q21	AF4	NTS, ATP/ GTP binding, Pro-Ser-rich	ALL, mixed AL
6q27	AF6	rod-like myosin homologue, GLGF motif	AML-M5, M4, ALL
9p22	AF9	NTS, ATP/GTP binding, Pro-Ser-rich, RNA polymerase II similarity	AML-M5, -M4, ALL
10p12	AF10	ZF and LZ "dimerization alone" motif	AML
17q21	AF17	ZF and LZ "dimerization alone" motif	AML-M5, -M3-like, mixed ALL
19p13	ENL	NTS, GTP-binding, Pro-Ser-rich, RNA polymerase II similarity	ALL and AML
Xq13	AFX1	Ser-Pro-rich	T-ALL

NTS, nuclear targeting sequence; Pro, proline; Ser, serine; GLGF motif, motif that is present in three proteins localized at cellular junctions and is associated with cytoskeleton; ZF, zinc finger; eps15, epidermal growth factor (EGF) receptor-pathway substrate 15; LZ, leucine zipper; "dimerization alone" motif, a ZF and LZ motifs without DNA binding potential. (Adapted from Bernand *et al.*, 1995.)

leukemias and 11q23 abnormalities have a poorer survival when compared to infants without 11q23 rearrangements. The poor survival is largely attributed to t(4;11) and t(11;19) translocations, while children with monocytic leukemia and t(9;11) translocation have a longer survival when compared to myeloid leukemias with other 11q23 translocations (Martinez-Climent *et al.*, 1995).

Other MLL rearrangements and rearrangements of other genes located on 11q23. Other rearrangements involving *MLL* gene in leukemias include insertion of a submicroscopic portion of chromosome 6, including part of AF6, into an apparently normal chromosome 11 (Poirel *et al.* 1996), 600-700 kb deletions of the 3' end of the gene distal to the MLL bcr in cases with t(9;11) and t(6;11) (Cherif *et al.* 1994), complex rearrangement consisting of inv(11)(q12q21) and deletion of the 11(q22-q23) (Abdelaal *et al.*, 1995), inv (11)(q14;q23) found in NHL; ins(10;11)(p11:q23;q24), inv ins(10;11) (p11:q23;q13), and ins(10;11)(p13:q23;q24) found in AML-M5 (Canaani *et al.*, 1995); and partial duplication of AML spanning exons 2-6, the part that is included in critical fused *MLL*-partner transcripts, found in AML patients with normal karyotype or trisomy 11 as a sole cytogenetic abnormality (Schichman *et al.*, 1994). Also, tumors of differing phenotype may have identical chimeric proteins, indicating that factors other that translocations involving *MLL* gene are contributing to the tumor phenotype (Correl *et al.*, 1993).

Some rearrangements of band 11q23 involve genes other than *MLL*. Thus, the t(11;14) (q23;q32) translocation has been identified in B-cell malignant lymphoma with two different breakpoints on 11q23. One breakpoint involved the p54/RCK gene, which encodes a helicase and the other breakpoint involved the still unidentified locus

centromeric to CD3D (Figure 5.1) (Bernard *et al.*, 1995). In another translocation, t(11;14)(q23;q11), TCRδ-gene was found rearranged with the presently unidentified gene telomeric of RCK. A new gene, PLZF, coding for a zinc finger protein located on 11q23 was identified after the study of a t(11;17)(q23;q21) translocation in an AML patient with rearrangement of the retinoid acid α-receptor (RARA) gene on 17q21 (Bernard *et al.*, 1995).

Mechanism of MLL rearrangements in secondary leukemia after epipodophyllotoxin chemotherapy. Between 2 to 12% of cancer patients who receive epipodophyllotoxins later develop sAML. In epipodophyllotoxin-related sAML the most common abnormality is the t(9;11) and the t(4;11) is the most common in the sALL (Domer *et al.* 1995). The majority of secondary epipodophyllotoxin-related leukemias are sAML, while secondary ALL make up only 6.8% of reported secondary leukemias (Domer *et al*, 1995). Epipodophyllotoxins (etoposide and teniposide) are non-intercalating topoisomerase II inhibitors. Topoisomerase II is a nuclear enzyme responsible for unwinding and relaxing the supercoiled DNA during regular processes of cellular metabolism (Liu 1989). This enzyme covalently binds DNA and introduces transient double strand breaks allowing another DNA helix to pass through the break. Such "cleavable complexes" are later resealed restoring the integrity of the DNA strands (Liu *et al.*, 1983). Epipodophyllotoxins block the resealing functions of topoisomerase II resulting in single and double strand breaks (Wozniak *et al.*, 1983, Long *et al.*, 1985). The non-random association of epipodophyllotoxins with chromosomal translocations at

11q23 band suggests that there are sites of unusual susceptibility to the effects of this chemotherapy within the *MLL* gene.

Balanced translocations, such as whose involving the *MLL* gene, represent an example of non-reciprocal genetic exchange, which might occur as a result of topoisomerase II-cleavable complex stabilization by epipodophyllotoxins. Support for such mechanism in leukemia induction comes from the identification of topoisomerase II DNA recognition sequences immediately adjacent (10-15 bp) to the breakpoints of the 11q23 rearrangement t(9;11) on both chromosome 11 and partner chromosome involved in leukemia (Bernard *et al.*, 1995). Interestingly, breakpoints actually occurred within heptamer sequences resembling VDJ recombinase recognition sequences in both *AF-9* and *MLL* genes. A complex mechanism involving other enzymes, in addition to topoisomerase II, has been implicated in the genesis of this translocation (Negrini 1993). Consistent with this hypothesis, etoposide was shown to induce aberrant recombination mediated by VDJ recombinase (Chen *et al.*, 1996). Sequences at the *MLL* and its reciprocal partner genes involved in rearrangements also showed homology to *Alu* repeats which are known "hot spots" for genomic recombination (Gu *et al.*, 1994, Negrini, *et al.*, 1993, Gu *et al.*, 1992). Strong topoisomerase II-mediated cleavage sites *in vitro* in normal *MLL* intron 6 were mapped in the vicinity (5 to 14 bp) of the translocation breakpoints in sporadic cases of leukemia (Felix *et al.*, 1995 (2)). Strong topoisomerase II *in vitro* cleavage site was also located 22 bp from the translocation breakpoint in the AF-9 partner gene of the t(9;11) translocation (Felix *et al.*, 1995 (2)). Insertion of three untemplated nucleotides at the t(9;11) breakpoint junction was also found. The slight misalignment of DNA topoisomerase II *in vitro* cleavage sites with respect to

translocation breakpoints and the untemplated insertions are consistent with topoisomerase II inhibitor-induced mutational spectra in other systems. Thus, small deletions and insertions of less than 20 bp at the sites of *in vitro* topoisomerase II-mediated cleavage were detected in the *aprt* locus of teniposide-treated CHO cells and acridine mAMSA- induced frameshift mutations were found at the DNA topoisomerase II *in vitro* cleavage sites in phage T4 (Han *et al.*, 1993, Ripley *et al.*, 1994, Ripley *et al.*, 1988). Of the proposed models to explain these observations, deletion of bases by a 3' exonuclease at the free 3' DNA end left by topoisomerase II, followed by insertion of a few untemplated nucleotides, would be consistent with the sequence findings at the t(9;11) translocation breakpoint junction (Ripley *et al.*, 1994, Felix *et al*, 1995 (2)). Topoisomerase II *in vivo* binding and cleavage have been demonstrated in the *MLL* breakpoint cluster region in HL60 cells (topoisomerase II cleavage sites occur every 20-300 kb in chromatin) (Domer *et al.*, 1995). Sequence analysis of the *MLL* breakpoint in sALL with t(4;11) (q21;q23) revealed 16/18 bp homology with the vertebrate topoisomerase II *in vitro* binding and cleavage consensus sequence located 4 bp centromeric of the breakpoint near *MLL* exon 9 and 15/18 bp homology with the topoisomerase II consensus 14 bp centromeric of the breakpoint in 4q21 (Domer *et al.*, 1995). The preferred site of topoisomerase II binding *in vivo* is favored in GC-rich core sequences surrounded by AT-rich DNA which was found in the *MLL* sequence just telomeric of the breakpoint in the sALL (Domer *et al.*, 1995).

FISH in the analysis of MLL rearrangements. Fluorescence *in situ* hybridization (FISH) has been used extensively in studies of *MLL* rearrangements. Lichter *et al.* (1990) mapped 50 cosmid clones to chromosome 11 with this method. Using their probes,

together with some additional probes, a more detailed map of 11q22-24 has been constructed (Kobayashi *et al.*, 1993 (2)). A variety of FISH probes is used now in the detection of *MLL* gene rearrangements, including cosmid probes surrounding the *MLL* bcr, YAC probe spanning the *MLL* bcr, and the whole chromosome 11 painting probe (Martinez-Climent *et al.*, 1995, Abdelaal *et al.*, 1995). Rearrangements in both *de novo* and secondary leukemias have been investigated (Super *et al.*, 1993, Zhang *et al.*, 1996, Martinez-Climent *et al.*, 1995, Poirel *et al.*, 1996). FISH allowed the identification of complex rearrangements and deletions involving MLL gene on 11q23 which could not be detected by standard karyotyping (Cheriff *et al.*, 1994, Abdelaal, *et al.*, 1995), and rearrangements in leukemic cells with apparently normal karyotypes (Martinez-Climent *et al.*, 1995). FISH showed that some of the cases with apparently normal karyotypes might go undetected as, for example, t(11;19) translocations which are difficult to detect by conventional cytogenetic analysis because only a small portion of chromosome 19 is translocated to 11q23 (Martinez-Climent *et al.*, 1995). Analysis of leukemias with cytogenetically detectable deletions of 11q13 to q23 has shown them to have a commonly deleted segment that is centromeric to the *MLL* gene suggesting the involvement of a putative tumor suppressor gene proximal to *MLL* (Kobayashi *et al.*, 1993 (1)).

Etiology of secondary leukemia. How epipodophyllotoxins induce human leukemias with translocations at chromosome band 11q23 is not known. Aplan *et al.* (1996) have identified a site within the *MLL* breakpoint cluster region that is highly sensitive to double-strand DNA cleavage induced by topoisomerase inhibitors *in vivo* in lymphoblasts of T-ALL patient, in various leukemic cell lines and in peripheral blood mononuclear

cells from normal volunteers. It has been proposed that topoisomerase II inhibitors induce site specific double-strand DNA breaks within the *MLL* bcr in normal hematopoietic precursor cells, and *MLL* translocations result from mistakes in the DNA repair process. The cells containing rearrangements within the *MLL* bcr then have a proliferative advantage conferred by the *MLL* translocations, and that proliferative advantage is clinically seen as a secondary leukemia (Aplan *et al.*, 1996). Recently, Kingma *et al.* (1997) have demonstrated that spontaneous DNA lesions such as apurinic sites, apyrimidinic sites, and deaminated cytosine residues poison human topoisomerase II α and stimulate cleavage proximal to leukemic 11q23 chromosomal breakpoints with an efficacy that was comparable to or greater than that of therapeutic concentrations of etoposide. These results suggest a potential role for endogenous topoisomerase II poisons in the initiation of leukemic chromosomal breakpoints.

A strong support for the hypothesis that the inhibition of topoisomerase II by the enzyme inhibitors used in chemotherapy is responsible for the induction of secondary leukemia would be a demonstration that treatment of cells with topoisomerase II inhibitors leads to the rearrangements involving *MLL* gene seen in secondary leukemias. In this study *MLL* FISH analysis of peripheral blood MNCs from 14 SCLC patients undergoing etoposide chemotherapy was performed. Results showed that the low sensitivity of the FISH assay is not sufficient enough to detect rare cells with *MLL* gene rearrangements if induced in patients after etoposide treatment.

3. Patients and methods.

Patients and cell lines. Blood samples were obtained after the informed consent from SCLC patients treated at the Victoria Cancer Clinic (Victoria, BC) between 1993 and 1995. The patients were usually treated with 4 to 6 monthly cycles of oral etoposide consisting of 50 mg tablets twice a day for 14 days separated by two weeks of rest. The number of cycles and total dose depended on individual patient's condition. The blood samples were usually drawn on the first day of each cycle before the drug was administered. The first sample was taken prior to treatment and was considered as a control for each individual patient. Information on 14 patients and 18 samples, which were available for the FISH study, is shown in Table 5.2. A total of 9 pre-treatment and 9 post-treatment samples were examined. Three patients (4E, 25E and 26E) were represented with both pre- and post-treatment samples. Other patients were represented with either only pre- or post-treatment samples. In one patient, 8E, two post-treatment samples, 8E-3 and 8E-4, taken after the third and the forth etoposide cycles respectively were analyzed. The time since the first and the last day of etoposide treatment prior to particular post-treatment sample varied as did the total etoposide dose administered to the patients (Table 5.2).

HL-60, a promyelocytic cell line derived from peripheral blood leukocytes, and RS4;11, an acute leukemia cell line established from the bone marrow, were purchased from ATCC (Rockville, MD). The RS4;11 cell line has the t(4;11)(q21;q23) and an isochromosome for the long arm of number 7, while HL-60 cell line has no rearrangement involving 11q23 chromosome band and served here as a negative control.

FISH. Digoxigenin-labelled MLL (11q23) DNA probe (Oncor, Gaitersburg, MD) was used in the FISH experiments. According to the manufacturer, the Oncor MLL DNA

Table 5.2. Information on 14 SCLC patients included in the FISH study.

Patient	Sex/Age	Pre-treatment sample	Post-treatment samples	Time from first day of treatment (months)	Time from last day of treatment (days)	Total etoposide dose (g)
1E	M/80		1E-2	1.7	14	2.0
2E[1]	F/62		2E-4	5	63	5.6
4E	M/83	4E-0	4E-1	0.1	3rd day of 1st cycle	0.3
6E	F/79		6E-1	0.3	9th day of 1st cycle	0.45
8E	M/68		8E-3	2.3	16	4.2
			8E-4	3.3	14	5.6
10E	F/77	10E-0				
15E	M/83	15E-0				
16E[2]	F/75	16E-0				
18E	M/78		18E-1	0.7	9	1.4
20E	M/68	20E-0				
25E	M/80	25E-0	25E-1	0.7	8	1.4
26E	F/73	26E-0	26E-1	1	15	1.4
27E	F/67	27E-0				
28E	M/71	28E-0				

Patients blood samples are indicated with -0 referring to the pre-treatment sample and with -1 (-2,-3 or -4) referring to samples taken after first (second, third or forth) etoposide cycles. Samples 4E-1 and 6E-1 were taken during the first etoposide cycle on the third and the ninth day respectively.

[1] Patient 2E had 4 courses of oral etoposide (50 mg twice a day for 14 days) and radiation therapy to the chest and head half a year prior to the time of sample 2E-4.

[2] Patient 16E was diagnosed with cancer of the breast (1983), cancer of the colon (1992) and SCLC (1994). No adjuvant treatment was administered to this patient prior to etoposide chemotherapy.

probe will give a normal *in situ* result (signal on both chromatids of both homologues) in samples which are normal, have a smaller deletion than is detected by the probe, point mutations, or other genetic etiologies. A non-deleted sample should show signal on both homologues with the MLL probe in 70%-90% of the cells analyzed. A deleted sample produces signal on only one homologue. An interphase or metaphase cell with a translocation will demonstrate three signals- a large signal on the normal allele or chromosome 11, which is sometimes visualized as a signal on each chromatid of the chromosome, and two smaller signals, one representing the MLL gene which has translocated or a signal on der (other) chromosome, and the remaining signal at the site of translocation or on the abnormal der (11) chromosome.

Cell harvest, slide preparation, hybridization and detection procedures were done according to manufacture's protocols (Chromosome *In Situ* Hybridization manual, Oncor, Gaithrsburg, MD).

FISH was performed on RS4;11 and HL60 cell lines which served as positive and negative controls respectively, on freshly isolated lymphocytes and whole blood samples from laboratory volunteers and on previously cryopreserved MNCs from patients. To obtain interphase nuclei and metaphases, log phase RS4;11 and HL60 cells were arrested and fixed prior to hybridization. PHA-stimulated lymphocytes grown for 72 hours were used in the experiments with freshly isolated blood cells. Whole blood samples were processed according to Quick Harvest Technique (Oncor, Gaitersburg, MD). Cryopreserved MNC fractions from patients samples were quickly thawed and incubated in supplemented RPMI 1640 (Gibco, BRL) (Chromosome *In Situ* Hybridization manual, Oncor, Gaitersburg, MD) for 24 hours to allow DMSO to exit the cells. After incubation

the cells were scored and processed according to cell harvest and hybridization protocols (Chromosome *In Situ* Hybridization manual, Oncor, Gaitersburg, MD).

Scoring. Slides were analyzed with Zeiss fluorescent microscope and the nuclei or metaphases were counted by eye. Scored cells were divided into 6 classes as having 1,2,3,4 or many dots and the last class consisted of uninformative cells with unclear morphology or ambiguous signals. Nuclei with no signal were not scored. Nuclei or metaphases with 1 dot included those cells with one dot or very closely located two dots in the case of replicated chromatids, which could not be interpreted as having two distinct signals. Nuclei or metaphases with 2 dots included cells with distinguishable two signals or four signals with two pairs of close dots in case of replicated chromatids. Nuclei or metaphases with three distinct spaced signals (some or all of them had double signal in the case of replicated chromatids) were counted as cells with three dots. Cells were assigned as having four dots if nuclei or metaphases had four distinct spaced signals. Cells with many dots were nuclei with more than 4 dots. These dots were usually of various intensity and cells with many dots were of significant number only on slides with excessive background or after amplification of FISH signal (Chromosome *In Situ* Hybridization manual, Oncor, Gaitersburg, MD).

Statistical analysis. Statistical analysis was done using Statistica software (Statsoft, Tusla, OK).

4. Results.

RS4;11 and HL60 cell lines. FISH analysis was performed on HL-60 cell line with normal chromosome 11 and on RS4;11 cell line with t(4;11) translocation involving *MLL* gene. From 199 to 693 interphase nuclei and metaphases were scored from each slide

(Table 5.3). It was observed that, while expected number of fluorescent signals appeared on the majority of cells from a given cell population, unexpected number of signals appeared on a small proportion of cells. Thus, HL60 cells sometimes appeared as having three signals, while some RS4;11 cells had only two signals (Figure 5.2). There was a small proportion of cells in both cell lines with either only one, four or many signals (Figure 5.2). There were also cases where the interpretation of the number of signals was hindered by the appearances of two signals located close together, so that they could represent either two signals on two different chromosomal regions or, alternatively, a split in two signal on two sister chromatids from the same chromosomal region (Figure 5.2 and 5.3).

Results showed that 81.4% of HL60 nuclei had 2 signals indicating the presence of unrearranged *MLL* gene and 71.5% of RS4;11 cells had 3 signals indicating the presence of rearranged *MLL* gene (a large signal on the normal allele, and two smaller signals , one representing the MLL gene which has translocated and the remaining signal on the abnormal chromosome 11) (Table 5.3, Figure 5.2). HL60 cells had 2.5% of nuclei with 3 signal (Table 5.3) (background "noise"), therefore more than 2.5%±3SD of cells need to have the *MLL* rearrangement in order for it to be detectable with this method. The hybridization in RS4;11 cells seemed to work not as good as in HL60 cells as RS4;11 sample had higher percentages of uninformative nuclei (16.6% *vs.* 8.5%) and nuclei with number of signals other than 3 (expected result from rearranged cells) (Table 5.3).

To evaluate the sensitivity of the assay in detecting the rearranged nuclei the serial dilution experiments were performed on 1/1, 2/1 and 5/1 mixtures of HL60/RS4;11 cells

Table 5.3. FISH results on HL60 and RS4;11 cell lines and their various dilutions.

Sample[1]	Total 1dot	Total 2 dots	Total 3 dots	Total 4 dots	Total many dots	Total uninformative	Aver. Cells/ field[2]	Total cell count	Fields count
HL60	10 5.0%	162 81.4%	5 2.5%	0 0%	5 2.5%	17 8.5%	3,5	199 100%	58
RS4;11	1 0.2%	38 9.0%	301 71.5%	6 1.4%	5 1.2%	70 16.6%	2,9	421 100%	147
1/1	1 0.4%	137 52.5% R= 1.6	85 32.6%	1 0.4%	2 0.8%	35 13.4%	5,3	261 100%	49
2/1	10 1.4%	392 56.6% R= 1.8	221 31.9%	6 0.9%	12 1.7%	62 8.9%	3,5	693 100%	196
5/1	4 1.0%	298 76.2% R= 6.8	44 11.3%	1 0.3%	0 0%	44 11.3%	3,2	391 100%	120

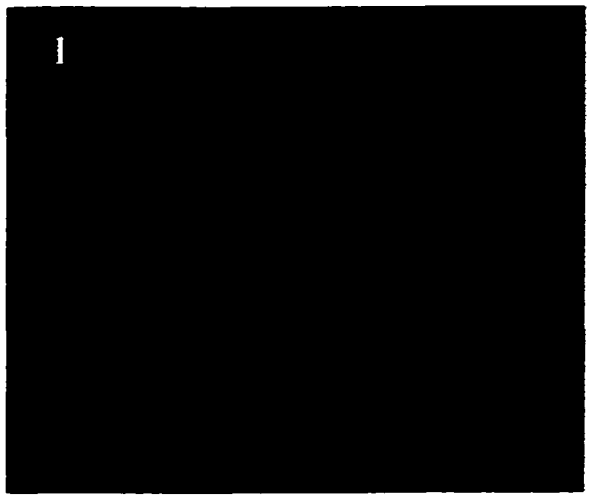
[1] Sample refers to HL60, RS4;11 cell lines or their dilutions. Dilution 1/1 is the dilution consisting of 1 part of HL60 and 1 part of RS4;11 cells; dilution 2/1 is the dilution consisting of 2 parts of HL60 and 1 part of RS4;11 cells; dilution 5/1 consists of 5 parts of HL60 cells and 1 part of RS4;11 cells.

[2] Fluorescent microscope with a 100x objective magnification lens was used for scoring. The number of cells seen in the microscopic field is shown.

R, ratio between the nuclei with 2 dots and nuclei with 3 dots.

Figure 5.2 FISH cell images showing fluorescence at the hybridization spots of the digoxigenin-labeled MLL (11q23) DNA probe.

1. HL60 cells showing two cells with two signals (expected) and one cell with three signals (unexpected aberration).
2. HL60 cell showing many signals (unexpected artifact).
3. HL60 cells showing two interphase cells and one metaphase spread with two signals each (expected). The signals are split in two between two sister chromatids on both chromosomes 11 in the metaphase spread.
4. RS4;11 cells showing two cells with three signals (expected) and one cell with what would be count as four signals (unexpected).
5. HL60/RS4;11 dilution 2/1 respectively showing one HL60 cell with expected two signals (one signal is split in two between two chromatids) and one smaller size RS4;11 cell with expected three signals.
6. RS4;11 cells showing metaphase spread with three expected signals. Each of the three signals is split in two between two chromatids of one normal chromosome 11, der(11) chromosome and der(4) chromosome.
7. RS4;11 cells showing two cells with three signals (expected) and one cell with two signals (unexpected). Some signals are split in two between two chromatids.
8. HL60/RS4;11 dilution 2/1 respectively showing two HL60 cells with expected two signals each and one smaller size RS4;11 cell with expected three signals. Some signals are split in two between two chromatids.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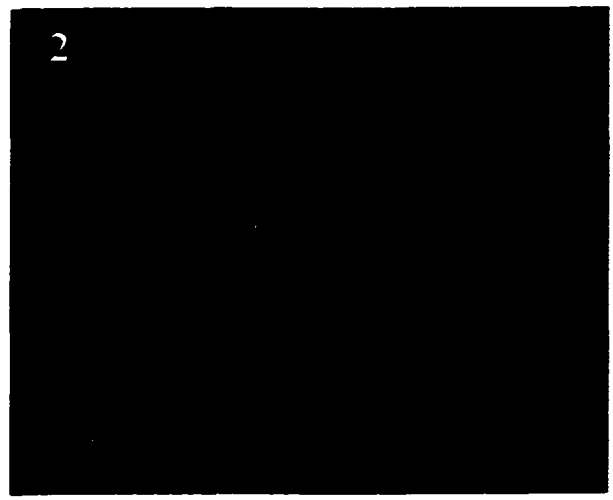
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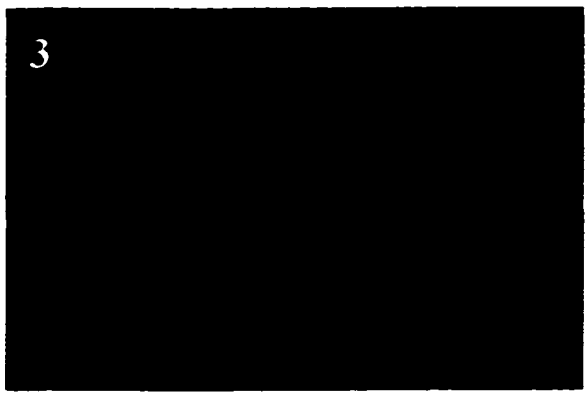
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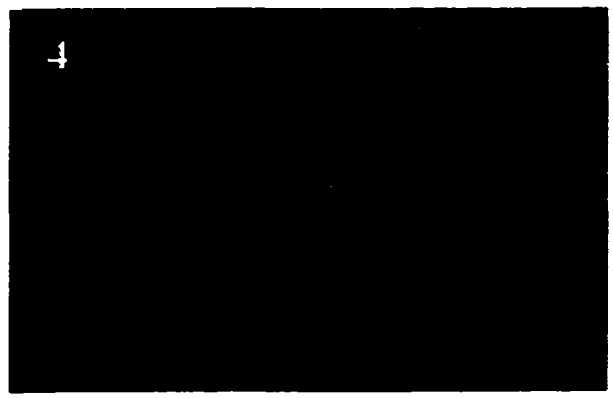
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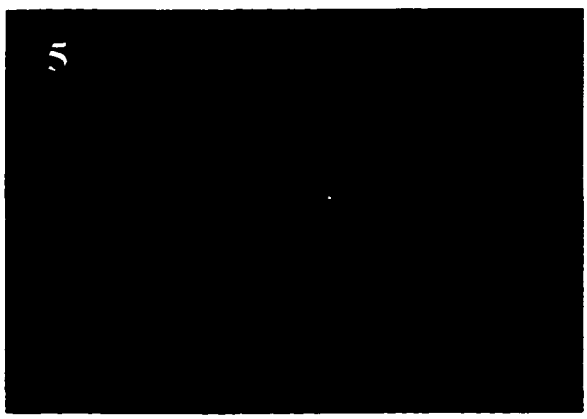
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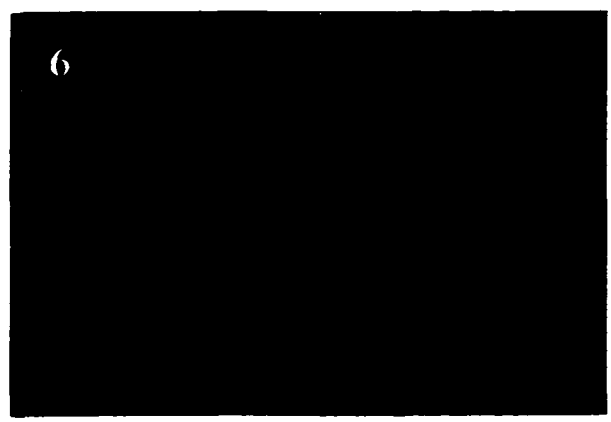
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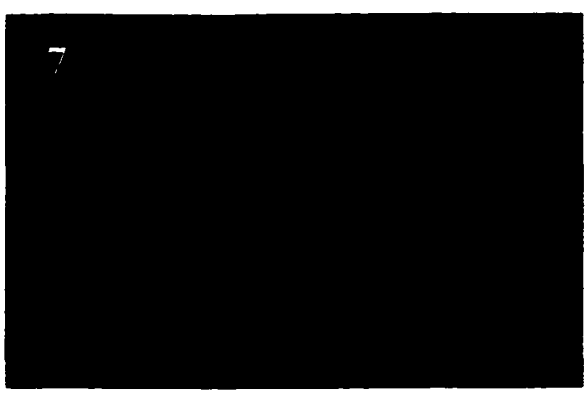
7


8

(Table 5.3). The ratios between the nuclei with 2 dots and nuclei with 3 dots were similar to the dilution ratios. The closest value of 1.8 obtained with a 2/1 dilution sample might be partially attributed to the large number of cells counted (693) compared to 1/1 (261 cells) and 5/1 (391 cells) mixtures (Table 5.3). The higher than expected ratios obtained for 1/1 (R=1.6) and 5/1 (R=6.8) dilution mixtures could be explained by the fact that hybridization in HL60 cells worked better with higher percentage of cells having informative signals. The percentage of uninformative cells in dilution experiments ranged from 8.9% to 13.4% which is in between the 8.5% for HL60 and 16.6% for RS4;11 cells (Table 5.3).

Statistical analysis determined that the distribution of 2- and 3-dotted cells was significantly different in 5/1 dilution mixture compared to that in HL60 cells ($\chi^2 = 12.6$, p=0.0004), in 1/1 dilution mixture ($\chi^2 = 49.3$, p=0.0000), and in 2/1 dilution mixture ($\chi^2 = 58.9$, p=0.0000). The distribution of 2- and 3-dotted cells was not significantly different between 1/1 and 2/1 dilution mixtures ($\chi^2 = 0.35$, p=0.5). Thus, while the 17% difference in the proportion of rearranged cells in 1/1 and 2/1 mixtures could not be detected, 16% difference in that proportion between 2/1 and 5/1 mixtures and 17% difference between HL60 cells and 5/1 dilution mixtures could be detected. Increasing the number of cells counted did decrease the discrepancy between the observed and expected values for the proportions of cells with various number of signals (Table 5.3).

Patients samples. FISH analysis of pre-etoposide treatment samples showed similar distributions of cells with various number of signals in 9 samples (Table 5.4, Figure 5.3).

Table 5.4. FISH analysis of 9 samples from SCLC patients before etoposide treatment.

Sample	Total 1dot	Total 2 dots	Total 3 dots	Total 4 dots	Total many dots	Total uninformative	Aver. cells /field[1]	Total cell count	Fields count
28 E-0	45	571	6	1	4	47	4,3	674	156
	6.7%	84.7%	0.9%	0.1%	0.6%	7.0%		100%	
4 E-0	51	581	5	0	0	101	4,9	738	150
	6.9%	78.7%	0.7%	0%	0%	13.7%		100%	
26 E-0	14	297	9	0	7	30	3,6	357	100
	3.9%	83.2%	2.5%	0%	2.0%	8.4%		100%	
25 E-0	12	623	24	3	29	70	3,8	761	200
	1.6%	81.9%	3.1%	0.4%	3.8%	9.2%		100%	
10 E-0	26	736	19	2	6	26	4,1	815	200
	3.2%	90.3%	2.3%	0.2%	0.7%	3.2%		100%	
27 E-0	23	963	4	0	1	45	5,2	1036	200
	2.2%	93.0%	0.4%	0%	0.1%	4.3%		100%	
15 E-0	41	931	8	1	2	28	4,0	1011	250
	4.1%	92.1%	0.8%	0.1%	0.2%	2.8%		100%	
20 E-0	15	755	4	2	1	9	3,9	786	202
	1.9%	96.1%	0.5%	0.2%	0.1%	1.1%		100%	
16 E-0	10	514	6	1	1	8	3,6	540	150
	1.9%	95.2%	1.1%	0.2%	0.2%	1.5%		100%	

[1] Fluorescent microscope with a 100x objective magnification lens was used for scoring. The number of cells seen in the microscopic field is shown.

Figure 5.3 Mononuclear cells from SCLC patients with FISH images showing fluorescence at the hybridization spots of the digoxigenin-labeled MLL (11q23) DNA probe. While majority of cells appeared as having two fluorescent signals (expected number from normal cells without 11q23 MLL gene rearrangements), a small proportion of cells appeared as having only one, three, four or many signals indicating an unexpected aberration or artifact.

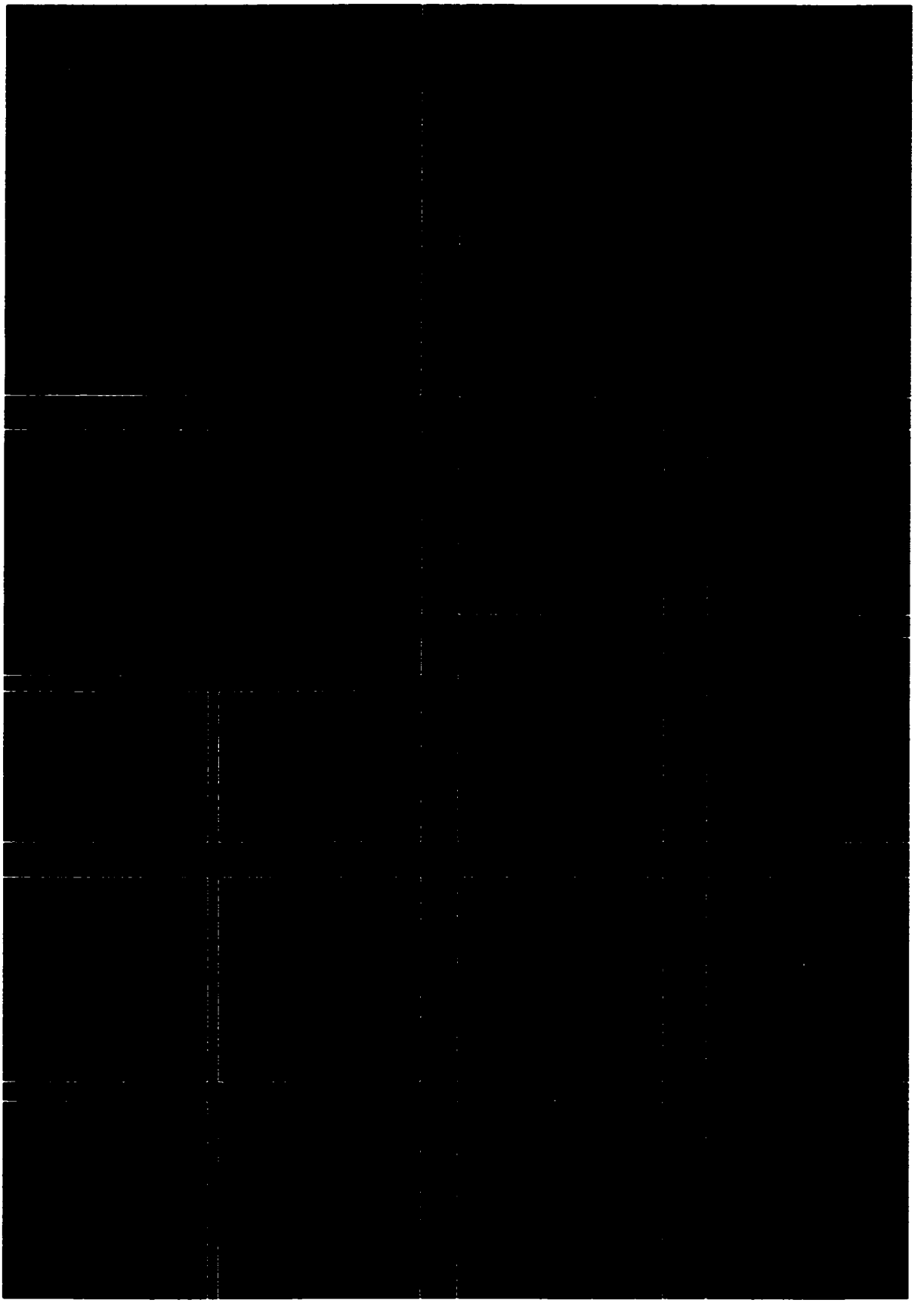

Table 5.5. FISH analysis of 9 samples from SCLC patients after etoposide chemotherapy.

Sample	Total 1dot	Total 2 dots	Total 3 dots	Total 4 dots	Total many dots	Total uninformative	Aver. cells /field[1]	Total cell count	Fields count
4 E-1	30	465	1	0	0	49	2,2	545	250
	5.5%	85.3%	0.2%	0%	0%	8.9%		100%	
1 E-2	26	254	4	0	0	71	3,0	355	119
	7.3%	71.5%	1.1%	0%	0%	20.0%		100%	
18 E-1	34	641	18	1	53	45	3,9	792	201
	4.3%	80.9%	2.3%	0.1%	6.7%	5.7%		100%	
26 E-1	26	499	13	0	18	41	3,0	597	201
	4.3%	83.6%	2.2%	0%	3.0%	6.9%		100%	
25 E-1	17	556	10	0	7	34	4,1	624	152
	2.7%	89.1%	1.6%	0%	1.1%	5.4%		100%	
8 E-4	12	549	1	0	0	54	3,1	616	200
	1.9%	89.1%	0.2%	0%	0%	8.8%		100%	
8 E-3	13	504	6	2	1	36	2,8	562	200
	2.3%	89.7%	1.1%	0.3%	0.2%	6.4%		100%	
6 E-1	22	662	6	0	0	52	1,7	742	430
	3.0%	89.2%	0.8%	0%	0%	7.0%		100%	
2 E-4	6	342	8	2	3	29	2,0	390	200
	1.5%	87.7%	2.1%	0.5%	0.8%	7.4%		100%	

[1] Fluorescent microscope with a 100x objective magnification lens was used for scoring. The number of cells seen in the microscopic field is shown.

From 540 to 1036 cells were counted from individual patients samples. Nuclei with 2 signals represented a mean of 88.4 ± 6.3% of cells and nuclei with three signals represented a mean of 1.4 ± 1.0% (Table 4). Cut-off level for the detection of the *MLL* rearrangement is estimated to be 4.4% (mean + 3SD).

FISH analysis of 9 samples taken from patients after etoposide treatment revealed results very similar to pre-etoposide samples (Tables 5.5 and 5.6). The mean proportion of nuclei with 2 dots in these samples was 85.1± 5.9% and the proportion of nuclei with 3 dots was 1.3± 0.3% (Table 5.5). None of the post-etoposide samples had the proportion of nuclei with 3 dots higher than the cut-off level of 4.4% (Table 5.5).

Table 5.6. Comparison of FISH results between 9 pre-etoposide and 9 post-etoposide samples from SCLC patients[1].

Treatment	N	Mean 1dot (%)	Mean 2 dots (%)	Mean 3 dots (%)	Mean 4 dots (%)	Mean many dots (%)	Mean uninformative (%)	Mean aver. cells /field[2]	Mean cell count
pre-etoposide	9	3.6 (2.0)	88.4 (6.3)	1.4 (1.0)	0.1 (0.1)	0.9 (1.3)	5.7 (4.2)	4.2 (0.6)	746.4 (212.1)
post-etoposide	9	3.6 (1.8)	85.1 (5.9)	1.3 (0.8)	0.1 (0.2)	1.3 (2.2)	8.5 (4.5)	2.9 (0.8)	580.3 (142.7)

[1] The numbers in parenthesis indicate standard deviation.
[2] Fluorescent microscope with a 100x objective magnification lens was used for scoring. The number of cells seen in the microscopic field is shown.

5. Discussion.

This study failed to demonstrate the presence of 11q23 *MLL* gene rearrangements in unstimulated peripheral blood lymphocytes of SCLC patients after etoposide chemotherapy using FISH with *MLL* DNA probe spanning the breakpoint cluster region. The negative result suggests that FISH technique is likely not sufficiently sensitive to pick up the rearrangements if they are induced in cancer patients receiving etoposide chemotherapy. FISH is very useful in identification of MLL rearrangements in leukemia patients where it is mostly done on bone marrow cells. In some leukemia cases leukemic cells can spread to the peripheral blood and, if their proportion is high enough (>10% usual cut-off level in normal cells), the presence of leukemic rearranged cells can be detected by FISH using peripheral blood cells from leukemia patients. SCLC patients studied here do not have leukemia and, therefore, it is not expected to find a substantial proportion of rearranged cells (>5-10% of cells detectable by FISH) in their peripheral blood. If cells with MLL rearrangement are induced by etoposide chemotherapy, their number is expected to be very small and more sensitive method such as RT-PCR appears to be a better choice for screening chemotherapy patients.

RT-PCR method for the detection of MLL gene rearrangements. Reverse-transcribed polymerase chain reaction (RT-PCR) techniques have now been widely used to detect the chimeric transcripts resulting from various translocations of the *MLL* gene Yamamoto *et al.*, 1994, Repp, *et al.*, 1995, Downing *et al.*, 1994, Hilden *et al.*, 1993). This method is based on the reverse transcription of total cellular RNA with random hexamer primers and subsequent PCR amplification of the cDNA *MLL* fusion transcripts. Because many

genes involved in the fusion with the *MLL* gene in leukemias are known, primers specific for the *MLL* gene (usually within the bcr in exon 5 or 6) and the partner gene surrounding the fusion point can be designed which would amplify the rearrangement. Sequencing of the PCR product can provide information on the exact location of the rearrangement breakpoints in both reciprocal genes. Primer sequences are available that can amplify the fusion points of rearrangements with t(4;11), t(6;11), t(9;11), t(11;19) translocations most commonly found in leukemias (Yamamoto *et al.*, 1994, Repp, *et al.*, 1995). Nested PCR primers were used to achieve maximum sensitivity.

RT-PCR analysis of the 11q23 rearrangements showed variability in specific fusion sequences. Thus, analysis of MLL/AF-4 fusion in cell lines and ALL patients revealed six different fusion junctions (Hilden *et al.*, 1993, Downing, *et al.*, 1994), all of which maintained an open reading frame. Three different breakpoints were found on *MLL* gene (between exons 6 and 7, 7 and 8, and 8 and 9) and three breakpoints were found on AF-4 (codons 347, 362 and 391 at the beginning of three exons). The areas of the *MLL* and AF-4 genes that were conserved in all der(11) fusion mRNAs and, therefore, likely to contribute to the function of the oncogenic fusion protein, are centromeric regions of *MLL* through exon 6 (retaining the AT hook motif) and telomeric regions of AF-4 beginning at codon 391 (containing nuclear localization and GTP-binding motif). In many cases more than one PCR products were detected due to alternative splicing and in one patient it was shown that either exon 7 or 8 of *MLL* gene could be fused to the AF-4 gene (Borkhardt *et al.*, 1994). RT-PCR characterization of *MLL* rearrangements in AML patients revealed rearrangements in 20% of AML M1 and in 60% of AML M5 subtypes (Poirel *et al.*, 1996). RT-PCR characterization of 11 cases showed four MLL self-fusions

(partial duplication of *MLL* exon 2 to exon 6), four MLL-AF6, two MLL-AF9, and one t(11;19) (Poirel *et al.*, 1996). Using various primer settings, chimeric mRNAs could be amplified in 6 of 7 leukemias with t(4;11), 6 of 8 leukemias with t(9;11) including secondary leukemia, 8 of 9 leukemias with t(11;19) and 1 with deletion at 11q23 which turned out to be an undetected by FISH t(11;19) translocation (Yamamoto *et al.*, 1994). A multiplex-PCR and fluorescence-based automatic DNA-fragment analysis was introduced which could discriminate between four different *MLL* rearrangements (partner genes on chromosomes 4, 6, 9 and 19) by a single PCR assay (Repp *et al.*, 1994). This method was applied for screening 50 patients with leukemia of different lineages and rearrangements were detected in five patients.

Comparison of RT-PCR and FISH methods. The superiority of the RT-PCR based methods compared to FISH analysis has been demonstrated. RT-PCR is sensitive to subtle translocations which could not be detected or may be misinterpreted by FISH analysis (Poirel *et al.*, 1996, Yamamoto *et al.*, 1994). Also, RT-PCR based method is much more sensitive than the FISH method. The sensitivity studies demonstrated that a single clone with chimeric mRNA in 10^4 to 10^5 cells or greater could be detected by RT-PCR (Yamamoto *et al.*, 1994, Downing *et al.*, 1994). At the same time, FISH analysis of cells from SCLC patients before treatment in our study demonstrated the cut-off level for the detection of rearrangements being 4.4% or 1 chimeric cell in 23 normal cells. RT-PCR strategy, on the other hand, will not detect rare or currently uncharacterized abnormalities. While the vast majority of breakpoints at 11q23 cluster at the 8.5-kb germline region spanning exons 5-11 of the *MLL* gene, breakpoints in partner chromosomes are heterogeneous (Yamamoto *et al.*, 1994). The use of a different

breakpoint or a different gene on the same region in the rearrangement will not be detected with the conventional primers. RT-PCR method is useful for the detection of the rearrangement where the partner gene is known prior to the analysis. More than 40 different chromosomal bands have been identified in the rearrangements with *MLL* gene in leukemia, however, primers for only 4 partner genes have been characterized. Although, these 4 partner genes are used more often in the rearrangements, the RT-PCR method is not suitable for the screening of patients for the appearance of the *MLL* gene rearrangements, as many of them would not be detected with primers specific only for some partner genes. *MLL* FISH analysis should theoretically detect rearrangements regardless of the partner, but frequent 3' telomeric deletions, *MLL* self-fusions, and interstitial insertions limit the use of this approach for screening purposes. While FISH method might be useful in doubtful or unidentified cases, or where confirmation of certain RT-PCR results is required, the RT-PCR method is better candidate for screening the cancer patients receiving etoposide chemotherapy for the appearance of presumably rare *MLL* gene rearrangements.

Chromosomal abnormalities in cancer patients after etoposide chemotherapy and proposed mechanism of secondary leukemia. Peripheral lymphocytes of SCLC patients receiving combination chemotherapy consisting of cisplatin and etoposide were examined for SCE frequency and chromosomal aberrations (Tominaga *et al.*, 1986). Significantly increased SCE frequency was observed in patients 3 or 4 days after treatment, however, it returned to the normal value as time elapsed after treatment. In addition, abnormal chromosome count and/or increased incidence of structural changes such as chromatid breaks and gaps, exchanges, dicentric chromosomes and fragments, were observed in

patients after treatment. Thus, etoposide chemotherapy clearly can lead to chromosomal aberrations in peripheral blood lymphocytes of cancer patients. Studies of etoposide in human lymphocytes showed that it can induce increased frequency of SCE and chromosomal aberrations such as breaks, exchanges and tetraploidy (Tominaga *et al.*, 1986). The clastogenic effect of etoposide has been investigated *in vitro* on lymphocytes of 5 healthy donors and non-random involvement of chromosomes 1, 11, and 17 in aberrations was detected (Maraschin *et al.*, 1990). These chromosomes are R-band-rich with actively transcribed genes, so they would have a high topoisomerase II activity and would be particularly susceptible to etoposide DNA breakage activity. In human lymphocytes exposed to etoposide an excess of reciprocal translocations was observed compared to the prevalence of deletions detected after exposure of lymphocytes to melphalan (Pedersen-Bjergaard *et al.*, 1994). Chromosome band 11q23 was one of several bands showing some propensity for rearrangement after etoposide exposure (Maraschin *et al.*, 1990).

The increased risk of sAML in long term survivors of topoisomerase II inhibitor chemotherapy evidences the genotoxic effect of the treatment. *MLL* rearrangements in secondary leukemias have been shown to highly correlate with prior therapy with topoisomerase II inhibitors (Rowley, 1993). Secondary leukemias after topoisomerase II inhibitor treatment (AML and less frequently ALL) have been shown to originate from immature haematopoietic progenitor cell (Zhang *et al.*, 1996). The t(9;11) cases are the most common, followed by t(11;19) and t(4;11) (Kobayashi *et al.*, 1995). The dividing cells in bone marrow on the way to their maturation are more likely to acquire the *MLL* gene rearrangement under the pressure of etoposide exposure than the non-dividing cells

in peripheral blood. It is possible that etoposide induces balanced translocations at topoisomerase II binding sites involving various chromosomal bands. This activity of etoposide is related to its ability to promote illegitimate recombination through the inhibition of topoisomerase II DNA breakage/religation activity. Among all rearrangements, translocations of MLL gene are perhaps most leukemogenic and, if induced in immature hematopoietic progenitor cell, such rearrangement, combined with secondary changes, may provide a proliferative advantage to the cell which clinically manifests as a secondary leukemia years after chemotherapy. It still remains to be determined if etoposide can directly induce specific 11q23 rearrangements of the MLL gene in human cells.

SUMMARY AND CONCLUSIONS

The genotoxic effects of single drug chemotherapy were investigated in 12 SCLC patients treated with etoposide and in 9 MM patients treated with melphalan. Results showed no increase in the mean *hprt* MF either in SCLC patients or in MM patients. This suggests that either the *hprt* T-cell clonal assay is not a sensitive enough method or that the chemotherapy does not induce *hprt* mutation in patients' T cells. Factors that might have contributed to the low sensitivity of the assay include small sample size and low CE that resulted in already high pre-treatment MF values and big 95% confidence intervals for the MFs. Time needed for the expression of mutations might also have contributed so that samples collected earlier or later in the course of the treatment might have been more informative.

Complex biology of T-cells and their limited life span are important factors needing to be addressed. More than 90% of circulating T-cells are in the G_0 phase of the cell cycle. Therefore, only a small percentage of all T-cells are potential targets for chemotherapy induced mutagenesis as DNA replication and cell division are needed in order for a mutation to be fixed. This effect is even stronger for cell cycle or phase specific drugs such as melphalan and etoposide respectively. Elimination of damaged and mutated cells by apoptosis may also have contributed to the negative result.

Despite the lack of an increase in the mean MF, a significant dose-dependent increase in the *hprt* MF was detected in one MM patient (5M). As this patient received the highest number of treatment cycles and the highest melphalan dose and, therefore, was followed

for the longest post-treatment time (23 months), it appears that dose might have a cumulative effect on the *hprt* MF.

Combined data from both groups of patients revealed ln (MF)= f(age, CE) relationships similar to those obtained from the unexposed population data combined from several laboratories. Thus, ln MF was independent of age in the 55-85 age group, lnMF was inversely proportional to the CE and CE was independent of age.

There was an overall similarity between the *hprt* mutational spectra from the pre-treatment, post-treatment patient's samples and the control unexposed population. There were, however, distinct significant changes detected in post-treatment spectra. In post-etoposide spectrum a significant enhancement of AT>TA was detected compared to the pre-etoposide and control spectra. There was also an increase in exon 1 splice mutants, primarily small deletions, and a concomitant reduction in exon 4 splice mutants. The significance of such changes is unclear as etoposide is expected to induce primarily large deletions and rearrangements due to its DNA strand breakage activity. In SCLC patients who were heavy smokers no increase of GC>TA transversions was detected compared to the unexposed controls. GC>TA transversions are associated with smoke carcinogen exposure and our result is consistent with earlier observations that smoking has no effect of the *hprt* mutational spectra.

Post-melphalan spectrum, on the other hand, differed from the control spectrum in the percentage of GC>TA transversions which was increased in the post-melphalan spectrum. This change is consistent with preferential guanine adducts and crosslinks by melphalan. Predominance of GC>TA and GC>CG transversions, most of which from

patient 5M, at the 5"GNC potential interstrand crosslink sites was also noticed in post-melphalan compared to pre-melphalan spectrum.

While in most samples clonality did not seem to have a significant effect on the *hprt* MF, in one etoposide patient (3E) a large *in vivo* expanded mutant clone was detected which led to highly elevated MF values. This expanded clone was found in pre-treatment as well as post-treatment samples taken 4 months later. While etoposide exposure did not seem to have a major effect on the persistence of the expanded clone, there is an indication that some cells from this clone might have accumulated additional mutations during the 4 month treatment period. Additional mutations were of a splice error type including intron sequence insertions. After correction for clonality, the MFs in this patients were reduced and fell into the normal range of other SCLC patients. Clonality studies also demonstrated that contrary to non-splice mutants, mutants with identical splice errors selected either from the same or repeat blood samples were unlikely to be clonal and, thus, were not contributing to the overestimation of the *hprt* MF.

During the course of clonality studies, sequencing of the PCR-amplified TCR-γ gene rearrangements from various *hprt* mutants revealed interesting features of this rearrangements. Thus, the use of rarely utilized variable genes V9 and pseudo-gene V7 in rearrangement was found in two mutants. In another mutant, a mixture of two variable genes was found in the rearrangement indicating that two alleles were rearranged differently. Additionally, two mutants selected from different etoposide patients were found to have identical rearrangements. These findings add important information to the understanding of T-cell biology.

Samples from SCLC patients were also screened for the appearance of *MLL* gene rearrangements often seen in sAML in patients previously treated with chemotherapy including topoisomerase II inhibitors. FISH with a probe spanning bcr of the *MLL* gene was used in this investigation. While important technique in leukemia diagnosis, FISH proved to be not sensitive enough to detect presumably rare *MLL* gene rearrangements that might be induced in cancer patients during etoposide chemotherapy.

In conclusion, these studies showed no genotoxic effect of cancer chemotherapy in the majority of patients studied, while prolong exposure to high doses of chemotherapy agents may be associated with an increase in mutation as measured by the *hprt* T-cell clonal assay. The studies also showed that the *hprt* mutational spectra might be more sensitive than the MF in mutagenicity studies in cancer patients. This study demonstrated specific changes in mutational spectra following chemotherapy. While significance of these changes is unclear at present, they might become useful when compared to yet unknown mutations relevant in secondary leukemia. While *hprt* T-cell clonal assay has been demonstrated to be useful in assessing the genotoxic damage in cancer patients following chemotherapy, T-cells might not be suitable for studying mutagenesis induced by cell cycle- and phase-specific drugs, as more than 90% of circulating T-cells are quiescent. The lack of an increase in mean MF in two groups of cancer patients after chemotherapy treatment seen in this study does not mean that these treatments do not result in genotoxicity in these patients. An increased risk of sAML implies that chemotherapy treatments induce substantial genetic damage to bone marrow that in some patients may develop into secondary leukemia. This is particularly relevant in survivors of childhood cancer. Study of specific chromosomal abnormalities seen in sAML with

highly sensitive methods might be useful in patients receiving chemotherapy. Combined mutagenicity and clastogenicity studies will provide better understanding of genotoxic effects of cancer chemotherapy in cancer patients and may identify those susceptible patients with the highest genetic damage and at the highest risk of developing secondary malignancies.

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