Microsatellite Instability and Aberrant V(D)J Recombination in Ataxia Telangiectasia Affected Family Members

by

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We accept this thesis as conforming to the required standard

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ABSTRACT

Ataxia Telangiectasia (AT) is an autosomal recessive disorder characterized by predisposition to cancers. AT heterozygotes, at increased risk for breast cancer, may comprise 6-8% of breast cancer patients. We investigated the use of aberrant V(D)J recombination to discriminate AT carrier status, and microsatellite instability as an indicator of genomic instability within the AT cohort, using PCR-based methodologies and genomic DNA. We report a wide variation in the frequency of aberrant hybrid T-cell receptors (TCR) within our study populations, a control group, AT homozygotes, heterozygotes, and relatives, accounting for no significant differences between our cohorts. Additionally, no microsatellite instability was observed as a result of germline alterations within the informative AT individuals. In conclusion, the frequency of aberrant hybrid TCR is not a useful indicator to discriminate AT heterozygous individuals. Due to the size of our study population, we do not rule out potential genomic instability within AT homozygotes and heterozygotes.

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Table of Contents

List of Tables

List of Figures

Acknowledgments

"Women may be the only group that grows more radical with age."

- Gloria Steinem

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Introduction and Thesis Rationale

At the commencement of this study we were interested in a possible genetic predisposition of individuals heterozygous for the genetic defect in radiation repair, Ataxia Telangiectasia (AT), to the development of breast cancer. Previous studies have shown an epidemiological correlation between increased risk for many forms of cancers when individuals carry two defective AT alleles (AT homozygotes) and specifically to breast cancer when individuals carry one defective AT allele (AT heterozygotes) (Swift *et al.*, 1987, 1991). Ataxia Telangiectasia is an autosomal, recessively inherited disorder. Individuals must inherit two defective alleles to express the symptoms of the disorder and AT heterozygotes appear phenotypically normal while still carrying the putative genetic defect or mechanism that puts them at increased risk for cancer. The Swift study (1987) looked at the prevalence of the AT defect, about 1 in 40,000-100,000 live births, and estimated from this data, that the carrier status in the general North American population is 1.4%. In terms of AT heterozygote involvement in breast cancer etiology, it could mean that 8-10% of breast cancer patients are carriers of the AT defect. More recently, molecular genotyping using microsatellite markers on the DNA of 775 relatives in 99 AT families and index-method statistical analysis put this estimate at 6.6% of breast cancer patients (Athma *et al.*, 1996). Even with the recent cloning of the *ATM* gene on chromosome 11q23 (Savitsky *et al.*,1995) there is no simple clinical test to determine AT heterozygotes in the population.

Our initial proposal was to use a series of cytogenetic and molecular assays in an effort to identify AT carrier status in a population of two hundred newly diagnosed breast

cancer patients in British Columbia. Through a collaboration with the BC Cancer Agency we planned to collect and bank both blood and tumour tissue samples from each patient. A comparison the results of assays performed on the peripheral blood lymphocytes (PBL) and the breast tumour tissue of these breast cancer patients may improve our understanding of the stepwise progression model of breast cancer development. The model is simple: any cell in an AT heterozygote receiving a "second hit" in the "normal" *ATM* allele would result in the loss of heterozygosity (LOH) and, therefore, loss of function. Alternatively, the second allele could be lost by a chromosomal event yielding LOH. In any case, any of its progeny would genotypically be AT homozygous. Consequently, tumour tissue from AT heterozygotes might be expected to have arisen as a result of such a loss of *ATM* function.

Unfortunately, our laboratory was unable to acquire matching blood and tissue samples for the vast majority of breast cancer patients. Despite this limitation the value of being able to identify AT heterozygotes in the population still holds considerable promise. Therefore, our investigation focused on the potential of identifying AT heterozygotes using two approaches. These were aberrant V(D)J recombination and microsatellite instability (MIN). This work was largely made possible because of blood samples from three British Columbian families with an AT-affected individual. We were able to obtain PBL samples from fourteen (14) people. These included , 4 homozygotes, 5 obligate heterozygotes and 6 first and second degree relatives whose AT status is unknown.

Our first assay was based upon a report of increased aberrant V(D)J recombination process in AT patients as well as some agricultural workers (Lipkowitz *et al.*, 1990; 1992). We investigated if the presence of a hybrid TCR containing Vγ- Jβ as described by

Lipkowitz *et al.* (1990) could identify AT heterozygotes in the general population. This may be especially important to women, since studies estimate the relative risk of breast cancer to be between 3.9 and 6.8 ($p= 0.006$) among obligate AT heterozygotes (Swift *et al.*, 1987; Morrell *et al.*, 1990; Swift *et al.*, 1991; Easton, 1994). The second assay was based upon microsatellite stability in an effort to detect germline microsatellite alterations in the AT family members.

The current understanding of the *ATM* gene, the genetic implications and clinical characteristics of AT-affected individuals are the subject of Chapter I. This chapter also includes basic information on the process of V(D)J recombination and it's implications on the aberrant formation of hybrid TCR. In addition, microsatellite markers which are becoming an important molecular tool in cancer research are discussed in relation to genetic instability in breast cancer.

Detailed methodology on all experimental procedures, including sample processing from collection and long term storage to T-lymphocyte culturing and expansion can be found in Chapter II. This Chapter also includes the biostatistical information on the donors. Several DNA extraction techniques were tried for optimal results and these are outlined as well. Both the V(D)J recombination and the microsatellite instability assay are polymerase chain reaction (PCR) based techniques, utilizing nested and touch-down PCR protocols, respectively. Agarose and polyacrylamide gel electrophoresis methods were used to resolve the results of PCR amplification. The results of both the V(D)J recombination assay and the microsatellite instability assay are outlined in Chapter III, while the implications and conclusions are discussed in Chapter IV.

Briefly, we conclude that measuring the frequency distribution of hybrid TCR, as a result of aberrant V(D)J recombination within 2 loci of chromosome 7, is not a useful approach to identify AT heterozygotes. Indeed, our observations do not support previous literature with respect to the distribution of this particular TCR rearrangement in the AT patient study groups. Moreover, we did not find the hybrid TCR to be a rare event in the control population. On the contrary, we report a wide variation in it's frequency within all our study groups. We speculate that the statistical differences that we found may be attributed to variations in individual metabolic rates in the clearance of environmental exposures, and even possible PCR artifacts from non-specific DNA amplification.

The study using microsatellite marker proved of limited use, although it was an assay readily implemented in the laboratory. We found no microsatellite instabilities (MIN) as a result of germline alterations within the informative AT individuals, though this finding may reflect the small sample size of our study population. Therefore, we do not rule out the possibility of excess genomic instability in AT homozygous and AT heterozygous individuals.

CHAPTER I - Background

1. The Ataxia Telangiectasia (AT) Defect

Two isolated and undiagnosed cases most likely to be Ataxia Telangiectasia were reported in 1926 and 1941. However, it was not until 1957 that three independent descriptions of the Ataxia Telangiectasia (AT) syndrome were described in the literature. Drs. Boder and Sedgwick presented a total of 7 cases to the medical community, while a pair of siblings were reported by Drs. Wells and Shy. In these earliest clinical cases, ***cerebellar ataxia***[1] and ***oculocutaneous telangiectasia*** were commonly reported features, hence the origin of the name (Miller, 1982; Gatti *et al.*, 1991). As case histories often included frequent ***sinopulmonary*** infections, Dr. Boder attributed the recognition of the disease in the late 1950's to the growing use of antibiotics which allowed AT-affected individuals to survive to mid-childhood when ocular telangiectasia is clinically manifested (Miller, 1982). Although incidence rates show geographic and ethnic variation, general rates are currently estimated between 1 in 40,000-100,000 live births (Swift *et al.*, 1976; Kaiser-McCaw, 1982; Swift *et al.*, 1986; Savitsky *et al.*, 1995).

1.1 Clinical Features

1.1.1. Neurological. Truncal ataxia is the initial clinical symptom, which can usually be detected at 1 to 2 years of age as AT-affected children have generally difficulty learning to walk (Gatti *et al.*, 1991; Woods and Taylor, 1992). However, AT may often be misdiagnosed as cerebral palsy or other neurological disorders. It is only when other

[1] Definitions for medical terminology are presented in the Glossary at the end of this dissertation.

clinical features, such as ocular telangiectasia, ***cerebellar***, and intention tremours, present themselves later in childhood that diagnosis is confirmed (Gatti *et al.*, 1991). Indications of other cerebellar disease are frequently present, including ***dysarthia*** and ***muscular hypotonia***, resulting in ***hypotonic facies and posture***, and drooling (Gatti *et al.*,1991). ***Cerebral ataxia***, displayed as a staggering gait and severe lack of muscular coordination, is progressively degenerative. AT-affected individuals will usually be confined to a wheelchair before their teen years (Gatti *et. al.*, 1991). ***Choreoathetosis*** as a prominent ***extrapyramidal*** feature presents in 90% of patients, while ***dystonia*** is a feature often found in adolescent and adult individuals whose progressive muscular atrophy of the spine continues into the thirties. In recent years, life expectancy has been increased from adolescence to survival into the fourth and fifth decades (Gatti *et al.*, 1991). At autopsy, pathological changes to the cerebellum involve degeneration of ***Purkinje cells***, including both quantity reduction and degeneration of the associated dendrites (Gatti *et al.*, 1991; Jackson, 1996).

Reports in the literature present conflicting information regarding mental retardation as a clinical feature of AT. Some publications even include progressive mental retardation as a characteristic feature of the syndrome (Friedberg, 1995; Jackson, 1996). However, Welshimer and Swift (1982) found no increased incidence of mental retardation and Gatti *et al.* (1991) reported that most patients have IQ scores in average range or above. In addition, the disease progression is accompanied by a "leveling off" of cognitive function rather than mental deterioration. Short term memory loss, suggestive of premature aging, has also been reported in patients in their twenties and thirties (Gatti *et al.*,1991).

1.1.2. Other Clinical Features. A hallmark clinical feature of the AT syndrome ***oculocutaneous telangiectasia***, generally appears by age five and progressively spreads in a symmetrical pattern. Telangiectasia is easily observed on the surface of the skin (Friedberg, 1995), as well, commonly found on areas that are exposed to the sun or subject to irritation (Gatti *et al.*, 1991). Although, the progression of oculocutaneous telangiectasia is considered the representative characteristic of ***progeric*** changes in AT patients (Gatti *et al.*, 1991), several other progeric changes are identified, including graying hair, ***senile keratosis***, and basal cell carcinomas of the face. Additionally, skin biopsies indicate morphological cellular changes similar to features found in aged individuals. Moreover, AT patients display abnormalities of the immune system, including increased T-lymphocyte rearrangements and either hypoplasia or absence of the thymus (Gatti *et al.*, 1991).

Since Ataxia Telangiectasia is an autosomal recessively inherited disorder, AT obligate heterozygotes show none of the clinical symptoms mentioned above because they still carry one wild-type allele (Welshimer and Swift, 1982). In any cell that this remaining allele became non-functional there would be a loss of heterozygosity (LOH) and, therefore, loss of function of the *ATM* gene. This cell and any of its progeny would genotypically be AT homozygous. Nevertheless, AT heterozygotes have shown increased incidence of ***idiopathic scoliosis*** and vertebral anomalies (Gatti *et al.*, 1991). To date, AT heterozygotes are not successfully detected at a clinical level. The value of detecting carrier status is important because there are increased health risks for these individuals; including increased sensitivity to ionizing radiation and predisposition to all types of

cancers, most specifically breast cancer (Swift *et al.*, 1976; Swift *et al.*, 1986; Pippard *et al.*, 1988; Morell, 1990; Athma *et al.*, 1996).

1.2 Molecular and Cellular Features

An outstanding cellular hallmark of Ataxia Telangiectasia is the hypersensitivity to ionizing radiation and radiomimetic compounds that generate double stranded breaks, such as bleomycin (Thacker, 1994). AT fibroblast cell lines were found to be three times more sensitive to cell killing by ionizing radiation than were fibroblasts from normal or AT heterozygous individuals (Hanawalt and Painter, 1985; Paterson *et al.*, 1985). It was originally thought ionizing radiation had such a devastating effect on AT cells because of a defect in their DNA repair mechanism, just as the nucleotide excision repair (NER) defect in xeroderma pigmentosum patients inhibits the repair of UV radiation (Shiloh *et al.*, 1985; Franks and Teich, 1993). An early report suggested that DNA repair deficiency was an explanation for the greatly elevated mis-repair of DNA strand breaks in AT patients (Cox *et al.*, 1982). Although AT cells demonstrated a lower rate of DNA synthesis than normal cells, including a longer S phase (Painter, 1985), to date no study has been able to find defects in DNA repair mechanisms. Hanawalt and Painter (1985) reported that X-rays produced the same spectrum of lesions in both normal and AT homozygous cells. Moreover, they demonstrated that AT homozygote and normal cells had the same rate of repair of both double and single DNA-strand breaks. The loss of a functional AT gene (*ATM*) is now characterized as a defective cellular response to DNA damage as is Bloom's syndrome and Alzheimer's disease (Friedberg, 1995).

Normally, in the presence of DNA damage, a fully functioning cell would interrupt the cell cycle to halt the synthesis of DNA to ensure sufficient time for DNA repair mechanisms to complete repairs. Two time points have been identified within the cell cycle, the G_1-S and G_2-M checkpoints, where cellular processes can be halted preventing the cells from advancing into the next phase of the cell cycle. The G_1-S checkpoint occurs in late G_1 in interphase, just prior to DNA synthesis in S phase, allowing repair to occur before errors are replicated and become "fixed" in the genome. The G_2-M checkpoint occurs in late G_2 in interphase, immediately prior to mitosis and consequently cell division. Once the cell has entered mitosis, it has committed itself to undergo cell division.

AT cells appear to be defective in their ability to detect DNA damage following ionizing radiation, and therefore fail to invoke the mechanisms that inhibit DNA synthesis. AT cells fail to halt cell cycle progression at both the G_1-S and G_2-M checkpoints after exposure to ionizing radiation, exhibiting radioresistant DNA synthesis (Painter and Young, 1980; Thacker, 1994). After exposure to γ-irradiation, normal human bone marrow progenitor cells as well as myeloblastic leukemia cells exhibited an induction of p53 protein which correlated with a transient G_1 arrest (Kastan *et al.*, 1991). The p53 protein is involved in a signal transduction pathway that controls cell cycle arrest in G_1 phase and programmed cell death, or apoptotsis, following DNA damage by ionizing radiation (Kastan *et al.*,1992; Canman *et al.*, 1994). In AT homozygotes there is a delayed induction or lack of induction of p53 in response to ionizing radiation (Kastan *et al.*, 1992).

AT heterozygotes have been found to show an intermediate sensitivity to ionizing radiation, which has been the focus for developing a screening tool to distinguish AT

heterozygotes from the general population. In an attempt to find a screening tool to identify AT heterozygotes in the general population, Paterson and colleagues (1985) reported the effect of chronic gamma irradiation exposure, rather than acute high dose exposure, on dermal fibroblasts. Acute gamma radiation exposure allowed AT homozygotes to be distinguished from AT heterozygotes and normals individuals, where as none of the obligate heterozygotes differed significantly from the normal controls. On the other hand, chronic gamma irradiation (0.03 Gy/hr for 50 hours, with an additional 16-20 hours for recovery of sublethal damage) allowed for weak discrimination of 66% (2 of 3) of AT heterozygotes (Paterson *et al.*, 1985). However, they concluded that this procedure was not practical as a routine screening tool being both labour intensive and time consuming, and offering only a small degree of discrimination power.

Early chromosome aberration studies using micronuclei as an endpoint to determine an individuals AT status produced conflicting results. Rosin *et al.* (1989) found increased levels of micronuclei in mucosal epithelial cells from the buccal cavity and urinary bladder of both AT homozygotes and AT heterozygotes. However, when lack of repair of potentially lethal damage (PLD) was measured in terms of increased micronuclei in fibroblast cell lines, AT heterozygote did not consistently exhibit increased micronuclei frequencies (Arlett and Priestley, 1985). Indeed, such result variability has made it impossible to consider micronuclei frequency as a screening tool to distinguish between AT heterozygotes and normal cells.

With respect to refined chromosome based routines, the literature suggests that the chromosomal "breaks and gaps" assay (Sanford *et al.*, 1990; Scott *et al.*, 1994), could detect an increased sensitivity to ionizing radiation in 25% of the population and that AT

heterozygotes would be included in this group (Spitz and Hsu, 1994). Although AT patients have clearly shown an increase in chromosomal abnormalities, including; chromatid gaps, breaks, fragments, dicentrics and chromatid exchanges, the individual responses are varied (Taylor, 1982). If the wide range of chromosomal damage response was due to differing exposures to environmental clastogens, then one would expect a clear relationship between chromosomal damage and age (Bridges and Harnden, 1982). Although AT heterozygotes have shown an increased sensitivity to ionizing radiation, a wide variation in individual responses prevents this from successfully discriminating AT homozygotes, AT heterozygotes and unaffected individuals within discrete groups.

In addition to increased chromosomal instability, cells from AT affected individuals have exhibited a reduced life span, as well as humoral or cellular immunologic defects (Franks and Teich, 1993). Reported immunodeficiencies include varying low serum levels of IgA, IgE, or IgG in individuals suffering from Ataxia Telangiectasia (Bridges and Harnden, 1982; O'Conner *et al.*, 1982; Gatti *et al.*, 1991). Elevated levels of alpha-fetoprotein (AFP) are also consistently found in AT homozygotes (Hecht and Kaiser-McCaw, 1982; Franks and Teich, 1993). An immunological and karyotyping study of two AT families reported the most frequent specific rearrangements in AT homozygotes involved translocations of chromosome 7 and 14 and were seen in both T-cells and B-cells (O'Conner *et al.*,1982). These specific anomalies involving chromosome 7 and 14 were not seen in the fibroblasts of AT homozygotes nor in the lymphocytes of the 2 obligate heterozygote mothers. As well, the AT heterozygotes exhibited normal levels of immunoglobulins and alpha-fetoprotein (O'Conner *et al.*, 1982).

1.3 Cancer Incidences

Non-neoplastic pulmonary disease is the single most common cause of death for AT-affected individuals, accounting for 46% of deaths. Neoplasias alone account for an additional 23%, while another 28% of deaths within the AT group involve both diseases simultaneously (Gatti *et al.*, 1991). The incidence of cancer in AT homozygous individuals is 100-fold higher than the general population (Athma *et al.*, 1996). Blood system malignancies, such as lymphoreticular neoplasms and leukemias, tend to predominate in preadolescent AT individuals. Solid tumours, including epithelial and brain tumours, predominate in both AT adolescents and adults (Gatti *et al.*,1991; Franks and Teich, 1993). In general, AT-homozygotes experience increased incidence of chronic lymphocytic leukemia (CLL), a B-cell malignancy typically characterized by a translocation involving the bcl-2 gene on chromosome 18q21(Leroux *et al.*, 1991).

For over ten years, epidemiological studies have implicated the individual carrier status as a causative factor in the increased risk of carcinogenesis. AT heterozygous individuals develop all types of cancers, including chronic lymphocytic leukemia and a number of solid tumours, such as breast, ovarian, bladder, stomach and pancreatic cancers (Swift *et al.*, 1976; Swift *et al.*, 1987; Pippard *et al.*, 1988; Swift *et al.*, 1990; Morrell *et al.*, 1990; Swift *et al.*, 1991; Easton, 1994). Although the relative risk for all cancers is increased by a factor of 3.1 for women and 2.3 for men in AT heterozygotes, the risk for the development of breast cancer alone is increased by a 5.8 fold for the AT heterozygous (Swift *et al.*, 1987; Gatti *et al.*, 1991). In addition, 6-10% of patients dying from ovarian cancer before the age of 55, gastric cancers before age 75, and blood malignancies (leukemia and lymphoma) before the age of 45, are estimated to be heterozygous for the

AT gene (Swift, 1976). In a study of the incidence of cancers in 44 families with AT, the relative risk for AT heterozygotes was estimated at 6.1 ($p \leq 0.005$), while the relative risk in 574 close blood relatives was 3.9 ($p \leq 0.01$) with breast cancer being the most frequent cancer site in this group. (Morrell *et al.*, 1990).

In hindsight, the main limitation of risk estimation studies is the lack of detailed family histories. This knowledge would allow for the accurate evaluation of whether the increased risk of carcinogenesis in AT heterozygotes follows a pattern similar to hereditary cancers. For example, in cohorts where extensive family histories were taken, the genetic pattern of breast cancer incidence could be traced to either familial or sporadic nature (Hulka and Stark, 1995; Claus *et al.*, 1996). Familial risk involvement is suspected if breast cancer patient histories include any of the following basic criteria, summarized by Hulka and Stark (1995), 1) two first degree relatives affected; 2) relative with bilateral breast cancer; or 3) close relatives diagnosed with cancer before the age of 40-45. Following the above criteria, early age at onset of breast cancer is the strongest indicator of familial disease whereas women at age ≥ 50 are considered passed the relatively high-risk period for hereditary breast cancer. When the probability of being a carrier for a breast cancer susceptibility gene was calculated in a population-based study using family histories of breast cancer, 7% of the general population are considered to be carriers of such a gene (Claus, *et al.*, 1996). Additionally, the highest proportion of breast cancers involving a susceptibility gene are believed to occur in younger women, with 33% of cases attributed to ages 20-29 years but only 2% of cases to ages 70-79 years.

More recently, the discovery of BRCA1 and BRCA2 genes confirmed a genetic link to breast cancer attributable to dominantly inherited susceptibility genes (Friedman,

1994). Mutations at the BRCA1 gene are responsible for about 4% of breast cancers in all age groups. This frequency increases to 25 % within cohorts of early onset, defined as age ≤ age 40 (Hulka and Stark, 1995). Considering that together, BRCA1 and BRCA2 are implicated in a small proportion of breast cancer cases, approximately 5% (Wooster and Stratton, 1995), the involvement of other genes in the role of breast tumour development cannot be ruled out. Other possible mechanisms, potentially involving genes other than BRCA1 and BRCA2, may be found responsible for a subset of breast cancer cases, currently defined as "sporadic", which encompasses up to 90% of all breast cancer cases.

New molecular evidence, using molecular genotyping of 775 individuals in 99 AT families, has been able to remove some of the uncertainty of these epidemiological studies (Athma *et al.*, 1996). They reported an overall increased risk for invasive breast cancer of 3.8 for AT heterozygous women. However, unlike the BRCA1 and BRCA2 genes, greater risk was not associated with early onset (premenopausal) of the disease. Even when increasing the determinant for early age of onset to 60 year of age, the odds ratio for the younger women was 2.9 ($p=0.009$), while women age 60 or older had an odds ratio of 6.4 ($p=0.002$). A further difference in the pattern of breast cancer exhibited in women carrying the AT gene, and women with the BRCA1 or BRCA2 gene, is the lack of evidence for increased frequency of bilateral breast cancer in AT heterozygotes (Athma *et al.*, 1996). In males, both the BRCA1 (Ford *et al.*, 1994) and BRCA2 (Gayther and Ponder, 1997) genes are associated with increased risk of prostate cancer. Although, to date, no such association of increased risk for prostate cancer has been reported in connection with the *ATM* gene.

2. The Mutated Ataxia Telangiectasia (*ATM*) gene

It was originally thought that a number of genes (genetic heterogeneity) was likely responsible for the development of the AT syndrome. However, linkage analysis mapped the *ATM* gene to chromosome 11q22-23 and identified four phenotypically identical complementation groups (Gatti *et al.*, 1988; Komatsu *et al.*, 1990; Ziv *et al.*, 1991), which showed the following distribution: Group A (55%), Group C (28%), Group D (14%) and Group E (3%). The complementation groups accounted for variable responses to ionizing radiation and radioresistant DNA synthesis within different cohorts of AT homozygotes (Mange and Mange, 1990). Recent molecular data have shown that the AT phenotype is due to mutation(s) in a single gene, namely *ATM*. This gene was found to be mutated in all Ataxia Telangiectasia individuals in 176 AT families (Savitsky *et al.*, 1995). To date, over 40 mutations have been identified in the *ATM* gene, the majority of which are predicted to lead to premature termination of the translation product and therefore to a truncated non-functional protein (Gilad *et al.*, 1996a). Wright and colleagues (1996) reported that 25/30 mutations, studied by their group, were unique mutations. The lack of site specificity suggested that several mutational spots in the gene can lead to truncated protein products. The high frequency of unique mutations would make population screening for ATM carrier status difficult with mutation-specific assays (Gilad *et al.*, 1996b; Wright *et al.*, 1996).

The function of the *ATM* gene remains illusive, however, its 12 Kb cDNA spanning 66 exons, shows a high degree of similarity (30-40%) to the phosphatidylinositol-3'kinase (*PI-3 kinase*) and to *TOR* genes in mammalian cells, as well as *TOR* homologs in yeast (Satvisky, *et al.*, 1995). The *TOR* and *PI-3 kinase* protein

products are involved in signal transduction pathways including the activation of T-cells by interleukin-2 (IL-2) and the G_1 phase of the eukaryotic cell cycle. Although only 19% identity with the *rad3* gene of the *Schizosaccharomyces pombe* was found, additional phylogenetic analyses have demonstrated that the *ATM* gene is highly related to both the *rad3* and *Tel*1 genes in *S. cerevisiae* (Satvisky *et al.*, 1995), as well as, the *mei*-41 repair gene in *Drosophilia* (Jackson, 1996). The *rad3*, *tel1* and *mei*-41genes are all involved in cell cycle control (Bentley *et al.*, 1996). Additionally, the *tel*1 gene is involved in regulation of telomere length, with mutations in this gene resulting in shortened telomeres (Greenwell *et al.*, 1995).

The ATM product is predominately nuclear expressed in a number of tissues and cell lines (Lakin *et al.*, 1996). Most recently, it has been suggested that ATM protein levels are modulated by post-transcriptional regulation, in response to environmental or cellular stimuli (Savitsky *et al.*, 1997).

3. The V(D)J Recombination Model

One of the bodies' chief defenses against foreign organisms is the generation of immune diversity through somatic cell rearrangement mechanisms specific to lymphocytes. This shuffling of gene segments that encode for proteins specifically involved in antigen recognition results in an almost infinite number of possible protein conformations (King and Stansfield, 1990). In the normal process, the V(D)J recombinase enzyme assists the random selection, breakage, deletion, and reassembly of various V (variable), D (diversity), J (joining), and C (constant) gene regions within each immunoglobulin locus in B-cells and T-cell receptor (TCR) locus in T-cells (Kendrew, 1994; Lewin, 1994). The T-

cell receptor loci encompasses genes for four polypeptide subunits (α, β, γ, δ) which normally form two types of TCR heterodimers, consisting of either αβ-subunits or γδ-subunits (Figure 1).

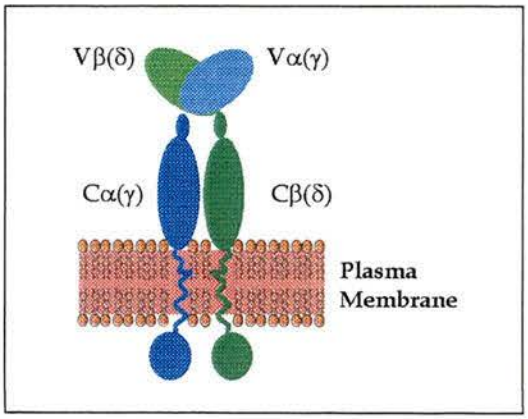

Figure 1. T-cell receptor model showing the normal heterodimer of αβ-receptor subunits, with the alternate arrangement of γδ subunits in parenthesis.

In the V(D)J reaction, the same structural format and mechanism is responsible for the physical rearrangement of gene sequences in both immunoglobulin and TCR gene rearrangements (Lewin, 1994). The V(D)J rearrangement elements are composed of two consensus sequences, consisting of a heptamer and a nonamer, which are presented in inverted orientation at each end of recombination sites (Figure 2). One member of each pair has a spacing of 12 bp between its components, while the other pair has 23 bp spacing. Recombining sites are present at the boundaries of all germline segments capable of participating in the joining reaction. The physical mechanisms of cleavage and rejoining occur as two separate reactions. Once the two heptamers help identify the coding ends of the genes, a double strand break at both heptamers completely releases the fragment

between the V and J-C coding ends. The two coding ends, one on each of the V and J segments, are covalently bonded in a coding joint that rejoins the DNA (Lewin, 1994).

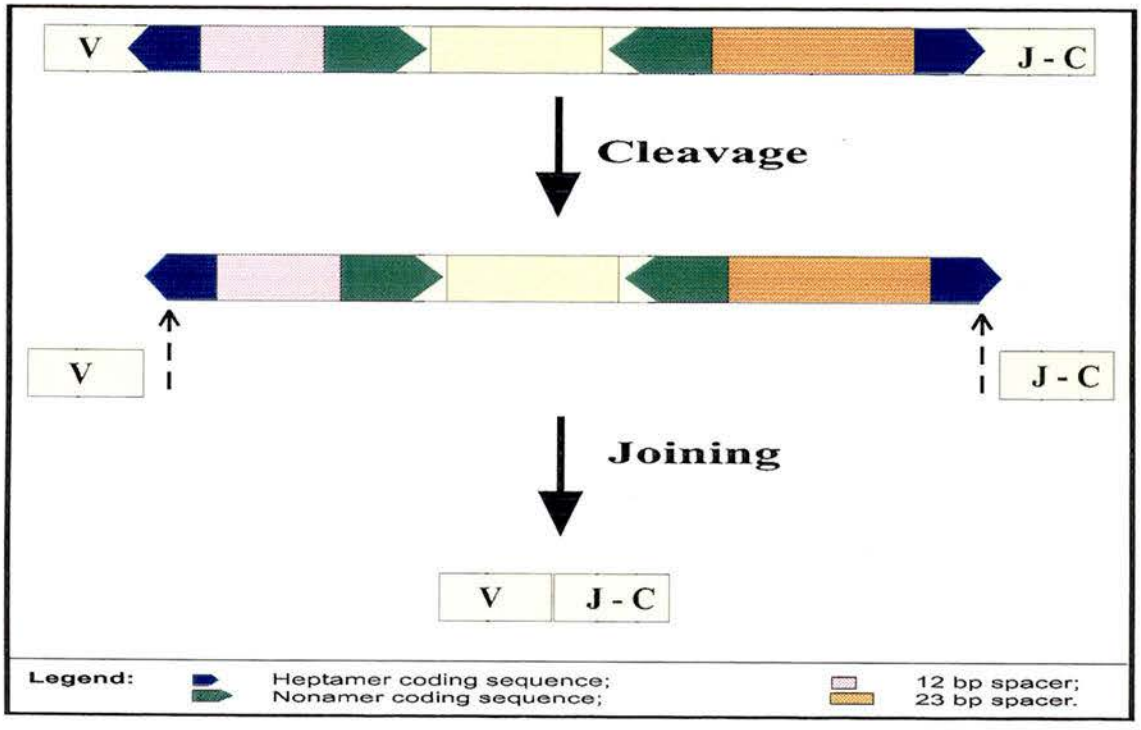

Figure 2. Elements involved in the cleavage and joining steps of V(D)J gene rearrangement.

It has been estimated that only one-third of recombinational events retain a correct reading frame (in-frame) and produce a functional gene product (Lewin, 1994). Since consensus sequences are on all immunoglobulin and TCR gene segments to increase the potential for immune diversity, some mechanisms must be in place to assist in productive, functional rearrangements. One such mechanism is the V gene promoter, found upstream of every V gene, which remains inactive until productively joined to a C gene region. Another mechanism is termed allelic exclusion, where productive gene rearrangement suppresses further gene rearrangement, resulting in a single type of immunoglobulin or TCR being expressed by each cell (Lewin, 1994). However, even

with mechanisms in place to guard the integrity of correct gene rearrangement, aberrant recombinations involving various TCR and immunoglobulin genes have been reported in a number of studies (Kojis *et al.*, 1989; Lipkowitz *et al.*, 1990; Gatti *et al.*, 1991; Lipkowitz *et al.*, 1992). These chromosomal rearrangements can produce successful in-frame aberrant hybrids.

Increased frequencies of aberrant hybrid TCRs, due to interlocus gene rearrangement, have been reported in patients with the AT syndrome (Lipkowitz *et al.*, 1990 and Kobayashi *et al.*, 1991), pesticide-exposed agricultural workers (Lipkowitz *et al.*, 1992) and patients with acute myeloid leukemia (Parreira *et al.*, 1992). It must be noted that the AT patient study populations of the Lipkowitz *et al.* (1990) and Kobayashi *et al.* (1991) were small, comprised of 5 and 2 individuals, respectively. Despite the population size, Lipkowitz *et al.* (1990) reported that the only AT heterozygote and the only unaffected AT sibling involved in the study exhibited a frequency of aberrant hybrid TCR similar to the control individuals.

As previously discussed, AT patients show greater numbers of chromosome breaks which appear as random events without preference to a particular chromosome (Hecht and Kaiser-McCaw, 1982). However, for breakpoints leading to chromosome rearrangements, the locations do not arise in a random fashion (Hecht and Kaiser-McCaw, 1982; Kobayashi *et al.*,1991). Most of the chimeric rearrangements in AT homozygotes involve chromosomes 7 and 14, predominately 7p14, 7q35, 14q12 and 14q32. Sites in chromosome 7 contain gamma and beta TCR genes while chromosome 14 harbours alpha-TCR and immunoglobulin genes, respectively (Gatti *et al.*, 1991; Lewin, 1994; Kendrew, 1994). Therefore, breakpoints in these specific regions are also involved in non-aberrant

chromosomal rearrangement processes in normal cells (Hecht and Kaiser-McCaw, 1982; Lipkowitz *et al.*, 1990).

In addition, chromosome rearrangements in AT homozygotes show possible tissue specificity. For example, the increase in balanced chromosome rearrangements that has been reported in lymphocytes of AT individuals was not observed in fibroblasts from the same individuals (O'Conner *et al.*,1982; Kojis *et al.*, 1989).

Although V(D)J recombination activity is not normally present in fibroblasts, Hsieh *et al.* (1993) provided essential components to activate the V(D)J recombination in fibroblasts to investigate inappropriate or inaccurate expression of components of the V(D)J recombination process. They found no evidence of abnormal V(D)J recombinase enzyme or its expression, nor did they find abnormalities in the signal and coding joint formation in the V(D)J reaction.

The main objective of this current study was to further investigate the involvement of aberrant gene recombinations resulting in aberrant hybrid T-cell receptors. Specifically, between variable regions on the gamma subunit (TCRVγ)[2], located at chromosome 7p14, and the joining region on the beta subunit (TCRJβ) located on chromosome 7q35 (Lipkowitz *et al.*, 1990) (Figure 3). Since the recombination process can utilize any of 13 functional Jβ segments on the gene (Concannon, 1992), different size PCR fragments, ranging in size from 230 bp to 2350 bp, can be generated (Lipkowitz *et al.*, 1990).

[2] Literature may use non-italicized nomenclature; products are simply listed as TCRVG and TCRJB.

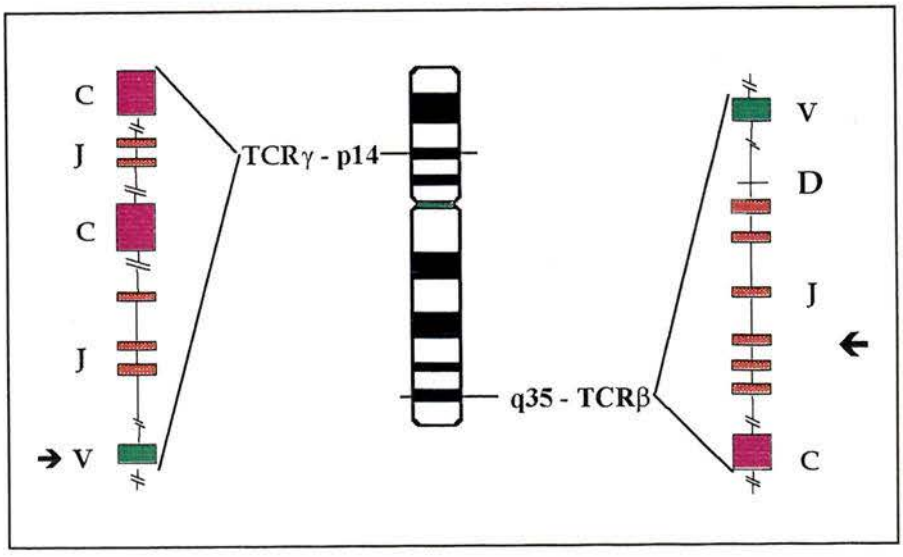

Figure 3. Aberrant intrachromosomal V(D)J recombination event Vγ- Jβ on chromosome 7. Arrows indicate the sites of one possible recombination that would produce a hybrid TCR.

It is estimated that approximately two thirds of hybrid V(D)J recombinations result in non-functional TCR polypeptides, as a result of a shift in the reading frame (Kirsh, 1994). The frequency of aberrant TCR in people exhibiting functional chimeric TCR was less than 5 Vγ-Jβ rearrangements/200,000 peripheral MNC of 95% of individuals tested and exhibit a 70 fold increase in AT homozygotes (Lipkowitz *et al*, 1990). This frequency is consistent with a cytogenetic study of inversion of chromosome 7 in "normal" individuals (Aurias, 1993), where the frequency of inversion was found to be <1 in 10,000 PHA-stimulated peripheral T-cell metaphases.

In a study evaluating the incidence of immunoglobulin and TCR(β/γ) gene rearrangement in 40 cases of acute myeloid leukemia (AML) using Southern blotting, initial results indicated 11 of 40 aberrant size fragments (Parreira *et al.*, 1992). However, further analysis with β and γ probes showed 9 of these "rearrangements" to be artifacts and actually germline configurations of the respective genes. Therefore, the investigators

concluded TCR gene rearrangement to be rare in these AML patients and not significantly more frequent in individuals with abnormal expression of lymphoid markers.

It has been suggested these interlocus recombination hybrids are not selected against in the thymus (Kirsh, 1994). There have not been previous reports on a thymus-clearance mechanism for aberrant V(D)J recombination events but some evidence for the accumulation of recombined DNA fragments exists and eludes to the possibility of their elimination in this tissue (Kobayashi *et al.*, 1991). In their study, aberrant hybrid PCR products, detected using Southern blot autoradiograms, were consistently higher in thymus tissue as compared to spleen, lymph node, bone marrow, and peripheral blood. Additionally, the intensity of bands seen in the thymus of unaffected individuals was the same as seen in PBL's of AT-affected individuals. However, the investigators cautioned that the findings could merely represent the relative differences in distribution of T-cells in various tissue or that the result represents the detection of only a subset of possible rearrangements.

4 Microsatellite Marker Applications

Microsatellites are simple tandem repeats comprised of over 20 tandemly repeated units of small sequences of up to 6 base pairs (Darvasi and Kerem, 1995; Marra and Boland, 1995), with dinucleotide repeats being the most abundant (Indraccolo *et al.*, 1995). Moreover, Poly(CA) repeats, the most common dinucleotide repeat sequences, may also allow conformational changes that enhance recombination events between DNA molecules (Indraccolo *et al.*, 1995). Although highly polymorphic, microsatellites are generally considered inherited in a stable Mendelian fashion, and, hence, used for genomic

mapping and DNA fingerprinting (Jeffreys *et al.*, 1985; Darvasi and Kerem, 1995; Indraccolo *et al.*, 1995). Although these repetitive sequences are occasionally found among the coding regions of genes, generally they are found within the non-coding regions and dispersed throughout the entire human genome (Valdes, *et al.*, 1993). It has been proposed that new alleles, due to differences in copy number of these repeat sequences, are formed during unequal exchange in meiosis or strand slippage in replication, with slippage being the predominant mode (Jeffreys *et al.*, 1985; Valdes, *et al.*, 1993). Recently, microsatellite alterations, identified by shifts on their electrophoretic mobility, have commonly been called microsatellite instability and have been shown to be markedly increased by mutations in DNA mismatch repair genes, as well as being associated with DNA polymerase slippage during DNA replication (Thibodeau et *al.*, 1993; Darvasi and Kerem, 1995).

It has been proposed that repeated nucleotide sequences may permit slipped-strand mispairing (SSM) and, therefore, be prone to insertions and deletions (Kunkel, 1990). In this slippage-misalignment model, newly synthesized DNA, complementary to a short direct repeat, slips relative to another short repeat on the strand that is being copied. The loop formation will result in either insertion or deletion of a few base pairs, depending on slippage occurring in the nascent or template DNA strand, respectively. Studies of frequently mutated genes in three human diseases; cystic fibrosis, β-thalassemia and hemopholia B, revealed that the number of deletions was 3 fold greater than the number of insertions (Darvasi and Kerem, 1995). For both insertions and deletions, mutations involving 1 and 2 base pair were the most common events.

The use of microsatellite alterations as a determinant of genetic instability has been reported in the literature since the late 1980s and has successfully been used as a tool in cancer research since 1993, especially in colon cancer research (Thibodeau, *et al.*, 1993), and some heritable neurological and muscular diseases (Mao *et al.*, 1994; Wooster *et al.*, 1994; Indraccolo *et al.*, 1995). Currently, hereditary non-polyposis colorectal cancer (HNPCC) a hereditary form of colon cancer, is recognized as due to widespread genetic instability, with both germline and somatic mutations in mismatch repair (MMR) genes (Mao *et al.*, 1994; Lui *et al.*, 1995; Parsons *et al.*, 1995). However, in sporadic colon tumours, only one of 10 patients exhibited microsatellite instability and also had a detectable germline mutation (Lui *et al.*, 1995). In addition, when four markers associated with human neurological diseases were used to detect cancers, the affected patients exhibited germline expansion of the particular repeat sequence (Mao *et al.*, 1994). Numerous microsatellite alterations were detected in DNA from non-neoplastic colon tissue and urinary tract epithelial cells in 3 of 4 HNPCC patients, with a greater prevalence present in the epithelial rather than connective tissue (Parsons *et al.*, 1995).

Large somatic expansions of short repeat sequences, particularly trinucleotide repeats, have been associated with several human diseases involving neuromuscular symptoms, including ***Huntington's chorea***, myotonic dystrophy and spinocerebellar ataxia type I (Patel *et al.*, 1994; Mao *et al.*, 1994; Wooster *et al.*, 1994; Darvasi and Kerem, 1995; Indraccolo *et al.*, 1995). Furthermore, the initial length of the repeat might influence the triggering of the genetic instability, with longer repeats having a higher probability of expansion (Indraccolo *et al.*, 1995).

In cancer research, the MIN assay is generally used to investigate somatic changes that have occurred when comparing normal tissue and tumour tissue from the same individual. Since we are investigating a pre-disease state in the AT family population, we are looking for evidence of germline alterations that may indicate a mutator phenotype, and therefore a predisposition to cancer. The mutator phenotype model was proposed in an attempt to address the multiple mutations found in many cancers (Loeb, 1991). Loeb acknowledges that the spontaneous mutation rate can adequately explain the two hit mutational model (Knudson, 1987) but that this model does not explain multiple mutations frequently associated with human cancers cells. If more than two mutations are to accumulate in a cell, Loeb suggests that the mutation rate within the tumour cells must be increased, either temporarily or permanently, above the spontaneous rate in normal cells. The reporting of microsatellite instability in tumour cells but not in corresponding normal tissue suggests evidence of a mutator phenotype (Loeb, 1994). Alterations within these tandem repeats may be an indicator of genetic instability in cancer cells.

Recently, investigators have been endeavouring to use microsatellite markers to detect germline instability as a precursor to specific cancers (Berthorsson *et al.*, 1995; Thrash-Bingham *et al.*, 1995; Arzimanoglou *et al.*, 1996; Borg *et al.*, 1996). In a study of genomic alterations in renal cell carcinomas, Thrash-Bingham and colleagues (1995) suggested that multiple mutational events may have arisen in premalignant cells. One individual exhibited two distinct tumours, a renal cell carcinoma and ***oncocytoma***, with a common microsatellite instability but with different loci involved in LOH. In a study of 46 sporadic colorectal tumours, microsatellite instability was found in 6 tumours, two of which had family histories of colon cancer suggesting a germline mutation. Indeed other

family members were found to carry the microsatellite instability, including malignant breast tissue from the mother of one of the probands (Bergthorsson *et al.*, 1995). However, in the investigation of microsatellite instability in 90 ovarian cancers, MIN was found in 3/28 (11%) of cases with a family history of ovarian cancer and 8/62 (13%) of cases without family history (Arzimanoglou *et al.*, 1996). With no germline mutations detected, Arzimanoglou and colleagues (1996) suggest that the genetic basis of microsatellite in ovarian cancer is different from that in HNPCC.

Genetic instability is thought to be a major factor in the initial steps to tumourigenesis and is considered instrumental in the progression to metastatic abilities and drug-resististant cells (Tlsty *et al.*, 1993). Although genetic instability does not necessarily lead to tumourigenesis, it may increase the likelihood of cancer development, as well as tumour cell heterogenetity which, in turn increases the likelihood of drug-resistance, metastatic ability, and decreased antigenicity (Tlsty *et al.*, 1993).

The *nm23-H1* loci at chromosome 17q21.3-q22 is located close to the BRCA1 gene (17q12-q21), however, it has been associated with an additional tumour suppressor gene (Hall *et al.*, 1992; Cropp *et al.*, 1993; Patel *et al.*, 1994). The *nm23 -H1* locus has been found deleted in 64% of sporadic breast cancers (Hall *et al.*, 1992) and its reduced expression has been associated with a metastatic phenotype and aggressive tumour progression (Leone *et al.*, 1991). In highly metastatic murine melanoma cells, transfection of murine *nm23-H1* resulted in a significant reduction in primary tumour formation.

On chromosome 11, loss of heterozygosity (LOH) is frequently seen at regions q23 and p15. In a study of 86 primary breast tumours, LOH was observed in 35% and 46% of tumours at regions 11p15 and 11q23, respectively (Winqvist *et al.*, 1995). Carter

et al. (1994) reported that in 40% of breast tumours tested LOH at 11q22 -23 was independent of LOH found on 11p15. Several reports have associated LOH of the region between D11S35 and APOC3 loci with several types of carcinomas, including breast, colorectal, ovarian and cervical, as well as, malignant melanoma (Litt *et al.*,1991, Bhattacharya *et al.*,1991; Carter *et al.*, 1994; Hampton *et al.*, 1994; Negrini *et al.*, 1995; Winqvist *et al.*, 1993; Winqvist *et al.*, 1995). Moreover, LOH of 11q22-23 has also been associated with a higher incidence of breast tumour progression to metastatic disease and decreased survival (Winqvist *et al.* 1995). In that same study, LOH at 11p15.5, the locus of the human Ha-*ras* gene, was not indicative of more aggressive cancer progression and none of the four tumours expressing LOH at only the 11p15 loci developed metastatic disease. The D11S35 and APOC3 markers are frequently lost in breast cancer and are in close proximity to the AT gene site at 11q22-23. Since our population involves AT patients and their relatives, and as we are ultimately interested in any possible contribution to breast cancer, it seemed appropriate to include both markers in our study. It should be noted that there have been reports against the linkage between breast cancer and the AT gene (Cortessis *et al.*, 1993). In a study involving 64 families with bilateral breast cancer, the *DRD2* microsatellite loci was found suggestive of linkage to the AT locus in only one family.

The p53 gene, a recessive tumour supressor gene, has long been functionally associated with the regulation of gene amplification, aneuploidy, cell cycle progression, apoptosis, and the suppression of cell transformation (Tlsty *et al.*, 1993). In addition, p53 is involved in solid tumours as well as hematopoietic tumours, the major type of cancers associated with AT patients (Tlsty *et al.*, 1993). Recently, the use of microsatellite loci has

allowed a better understanding of the involvement of the p53 tumour suppressor gene in the development of breast cancer (Sato *et al.*, 1990; Sato *et al.*, 1991; Futreal *et al.*, 1992; Cawkwell *et al.*, 1994). Perhaps the most persuasive arguments for selecting the p53 marker for our study are: 1) p53 loss or mutation is the most common alteration found in sporadic, nonfamilial cancers, including breast cancers (Vogelstein, 1990; Osbourne *et al.*, 1991;); 2) the PI-3 kinase, defective in AT individuals, may have a direct activation role in the DNA-induced p53 checkpoint (Guidos *et al.*, 1996; Morgan and Kastan, 1997); 3) the persistence of coding ends formed during V(D)J recombination reactions may also activate the p53-dependent checkpoint; and finally (Guidos *et al*, 1996), 4) there is a strong correlation between LOH at 11q22-23.3 and LOH at the p53 locus, in breast tumours (Carter *et al.*, 1994). Of 62 tumours assayed, LOH on chromosome 11 was found in 25% (7 of 28) of informative cases at marker D11S35 (Carter *et al.*, 1994). Additionally, 90% (9 of 10) of breast tumours with genetic loss at 11q22-23.3 displayed LOH at 17p13.3 (Carter *et al.*, 1994).

Both *Ank1* and D8S135 loci are localized at chromosome 8p where the DNA β polymerase repair and replication gene is also located. Dinucleotide deletions were most frequently seen at the *ANK1* marker, occurring in 5 of 13 breast tumours, while the D8S135 was associated with insertions in 5 of 13 tumours (Patel *et al.*, 1994). Instability at these markers was independent of normal indicators of tumour progression, such as: stage, metastasis, estrogen, or progesterone receptor response, suggesting that genomic instability may be an early event in tumourigenesis.

The D6S105 locus is associated with the human leukocyte antigen (HLA), the designation for the human major histocompatibility complex (MHC) (Weber *et al.*, 1991).

HLA molecules are highly polymorphic surface proteins expressed on the surface of most cells and specifically define individual tissue types. Additionally, antigen recognition in lymphocytes requires the formation of a trimolecular complex involving the HLA, the TCR and a peptide antigen that can be bound by the other two elements (Concannon; 1995; Kendrew, 1994). The D6S105 marker has been associated with inherited disease susceptibility, principally ***hemochromatosis*** (Beutler *et al.*, 1995; Roberts *et al.*, 1997). This locus may be involved in site specific alterations, and, therefore, a useful measure of potential mutations at both the TCR and HLA regions.

CHAPTER II. Materials and Methods

1. Donors and Sample Collection

Peripheral blood was collected into sterile Vacutainer Cell Preparation Tubes (Becton Dickinson) containing sodium heparin. Immediately after collection, the tubes were inverted 5-6 times to mix and then left upright and allowed to come to room temperature, approximately 30 min, and cell separation procedures followed the manufacturer's instructions. If it was necessary to store the blood samples (< 24 hours) in order to transport them before separating the mononuclear cells (MNC), then the cells were resuspended into the plasma and an alternate separation protocol was used as outlined by the manufacturer.

MNC's were separated by centrifugation for 30 minutes in a Sorval T6000 (DuPont), with a horizontal rotor, at 1500 to 1800 relative centrifugal force (RCF). The whitish layer of mononuclear cells and platelets sitting just above the polyester gel separation media was collected with a Pasteur pipette and transferred into a 15 ml conical centrifuge tube. The cells were washed in blank RPMI, counted and then resuspended in Freezing Medium [45% FBS (Gibco), 45% RPMI and 10% DMSO (Sigma)] at a concentration of 2-5 x 10^6 cells/ml and aliquoted into cryopreservation vials. To reduce damage to the cells, the vials were placed in a styrofoam container in the -80°C freezer and allowed to freeze at an initial rate of -1°C/minute for the first 24 hour period before transferring to liquid nitrogen storage.

1.1. AT Study Population

Peripheral blood was collected on a voluntary basis from 15 individuals, representing three AT families in British Columbia, Canada. The participating groups included 4 AT homozygotes, 5 AT obligate heterozygotes and 6 AT family relatives, with unknown AT-carrier status. The individual pedigrees are depicted in (Figure 4). Biostatistical information, including age and sex distribution for our study groups can be found in Table 1. Although two of the AT families, identified as F1 and F2, are related, consanguinity is not an issue since the fathers of these families are brothers. Both AT heterozygous men married unrelated women who unknowingly carried the *ATM* gene. As a result, the two couples had AT affected children, F1P1 and F2P1 respectively. Both AT heterozygote mothers (F1H1 and F2H1) and one of the fathers (F1H2) donated blood samples. AT patients F3P1 and F3P2 are siblings in the family identified as F3 who are not related to AT families F1 and F2. At last the six AT relatives are donors in which the AT-carrier status is unknown (UN) consisting of non symptomatic family members of F1H1.

Figure 4. Genetic pedigree for three Ataxia Telangiectasia families in British Columbia. Two of the families, F1 and F2, are related, while F3 is not related to these families. The AT Relatives are extended family members of F1H1.

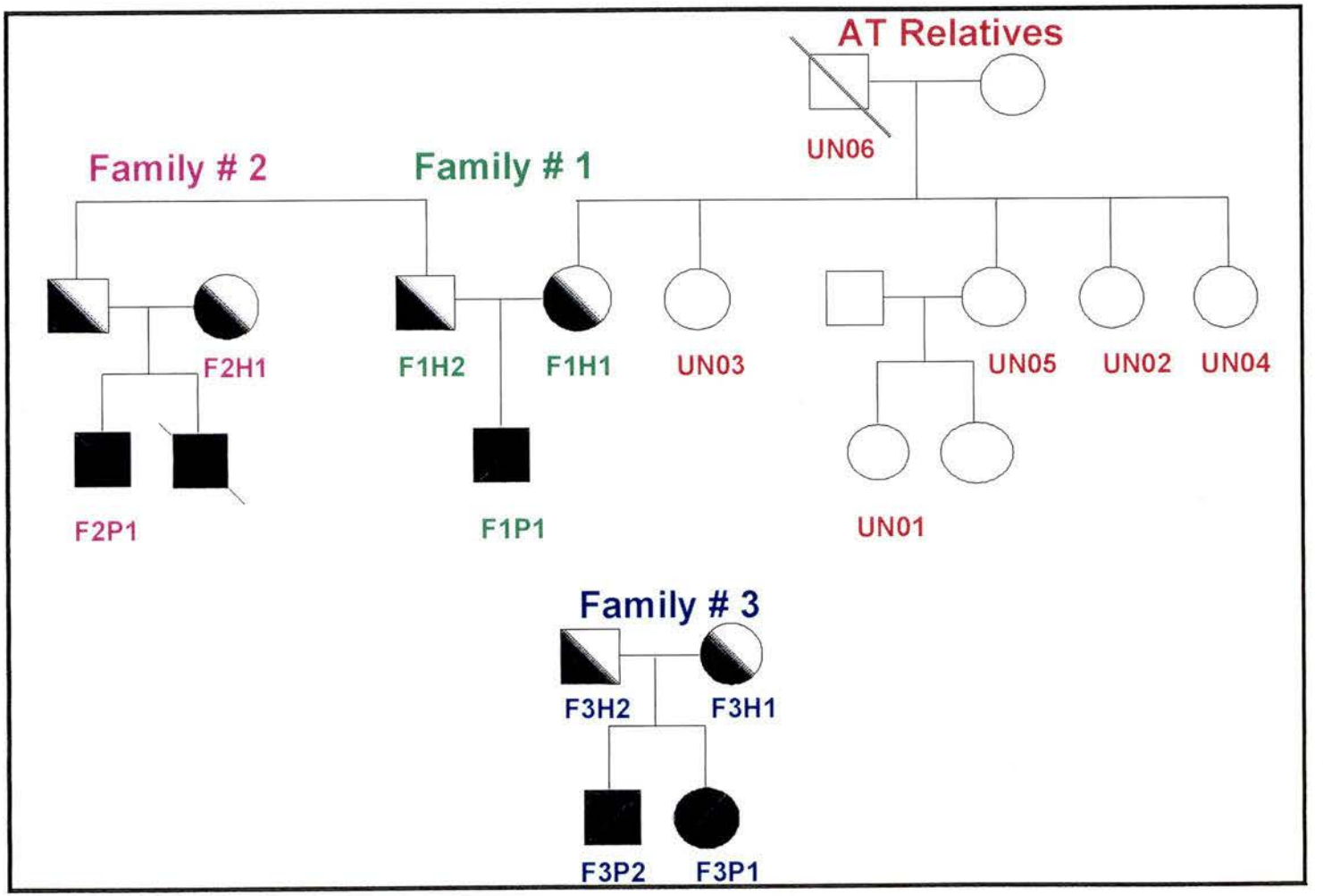
AT Relatives
UN06
Family # 2
Family # 1
F2H1
F1H2
F1H1
UN03
UN05
UN02
UN04
F2P1
F1P1
UN01
Family # 3
F3H2
F3H1
F3P2
F3P1

Table I. Biostatistical information on participating donors of three AT Families in British Columbia, Canada included in this study.

Donor ID	Age (years)	Sex	Smoking/ Non-smoking	Vegetarian/ Non-veg.
AT Probands (n=4)				
F1P1	8	M	NS	NV
F2P1	24	M	NS	NV
F3P1	7	F	NS	NV
F3P2	5	M	NS	NV
AT Heterozygotes (n=5)				
F1H1	44	F	NS	NV
F1H2	54	M	NS	NV
F2H1	43	F	NS	NV
F3H1	46	F	NS	NV
F3H2	47	M	NS	NV
AT Relatives (n=6)				
UN01	11	F	NS	NV
UN02	39	F	NS	NV
UN03	42	F	NS	NV
UN04	37	F	NS	NV
UN05	41	F	NS	NV
UN06	78	M	NS	NV

1.2. Unaffected Control Population

For the V(D)J recombination study in AT family members, peripheral blood was collected from 25 healthy individuals who were not affected by the AT disorder and had not been diagnosed with breast cancer (Table II).

2. Short Term T-lymphocyte Culture and Expansion

The protocol for culturing the AT primary lymphocytes was adapted from O'Donovan, *et al.* (1995) which uses Dutch Modified RPMI 1640 with 10% FBS and freeze-killed feeder cells. We modified O'Donovan's protocol by using RPMI 1640 and 15% fetal bovine serum (FBS) and the traditional lethally irradiated feeder cells (50 Gy).

Samples were taken from liquid nitrogen storage, thawed at 37°C and washed in blank RPMI 1640. The cells were then centrifuged at 1000 rpm for 9 minutes, the

supernatant was removed and the cells resuspended in Stimulation Medium [15% FBS, 20% HL-1, 4% antibiotic solution, 0.25 μg/ml phytohaemagglutinin (PHA) (Murex Diagnostics Limited)]. The antibiotic mixture contained 0.6 g penicillin (Sigma), 1.0 g streptomycin (Sigma), 2.92 g L-glutamine (Sigma), 2.0 g sodium pyruvate (Sigma) and 400 ml of double distilled water.

Table II. Biostatistical information for individuals used in the control group for the V(D)J Recombination Assay.

Donor ID (n=25)	Age (years)	Sex	Smoking/ Non-smoking	Vegetarian/ Non-veg.
BC96	19	M	NS	NV
BC97	25	F	NS	NV
BC98	18	M	NS	NV
BC107	42	F	S	NV
BC112	42	F	NS	NV
BC113	25	M	S	NV
BC115	43	F	NS	NV
BC116	33	F	S	NV
BC117	26	F	S	NV
BC119	37	F	NS	NV
BC122	14	M	NS	NV
BC123	9	F	NS	NV
BC124	10	F	NS	NV
BC125	6	M	NS	NV
BC127	24	M	NS	NV
BC130	28	M	S	NV
BC139	26	M	NS	NV
GN01	4	M	NS	N/A
GN02	child	M	NS	N/A
CN01	34	F	NS	NV
CN02	44	M	NS	NV
CN03	31	F	NS	NV
CN04	28	M	NS	NV
L2A	70	M	NS	NV
L39-1	65	M	NS	NV

Lethally γ-irradiated RJK cells were added to the primary cell suspension to a concentration of 10^5 cells/ml. Prior to being used, feeder cells were lethally irradiated with a dose of 50 Gy in a Gamma Cell 1000 irradiator (Nordion International Inc.). The

presence of feeder cells has been shown to provide antigenic stimulus and cytokines to T-lymphocytes, enhancing stimulation by PHA (Fleischer, 1983; O'Donovan, *et al.*, 1995). RJK-853 cells are a lymphoblastoid cell line derived from a Lesch-Nyhan patient in Toronto with a complete deletion of the *hprt* gene. The RJK feeder cells were continuously cultured in growth medium [10% CBS (Gibco/BRL), 4% antibiotic solution, and 86% RPMI] at concentrations of approximately 2.5 x 10^5 cells/ml.

The cell suspension, containing both primary T-lymphocytes and RJK feeder cells in complete growth medium was plated into 6-well plates (5ml/well) and incubated at 37°C, 5%CO_2 with constant humidity for 4 days. On the 4^{th} day and daily thereafter, they were disaggregated by pipetting. When the cell concentration reached approximately 1-2 x 10^6 cells/ml, wells of the same sample were pooled and diluted 1:1 with fresh Growth Medium, containing 15% FBS, 20U/ml IL-2 and 4% antibiotic solution but no PHA. The new cell suspension was transferred to a 75 cm^2 tissue culture flask and kept in culture until the cell concentration reached 2 x 10^6 cells/ml, when cells were harvested for either cryopreservation or DNA extraction (O'Donovan, *et al.*, 1995).

3. DNA Extraction Techniques

Three DNA extraction techniques are described throughout this dissertation, namely Phenol/Chloroform, Dialysis DNA Isolation Method (Rogers *et al*, 1996), and the Wizard Genomic DNA Purification Kit (Promega). Despite the common use of phenol/chloroform to extract and purify DNA, the amount and quality of DNA extracted was found to be very inconsistent. Since samples were, at best, very difficult to replace and, in some cases, irreplaceable, we decided to use more consistent procedures. In

general, the dialysis method resulted in very good DNA. However, it required very large numbers of cells (5-10 x 10^6 to 10^7 cells) and it was more laborious and more time consuming, with a 24-48 hour dialysis time. The Wizard kit gave good and consistent results, was easy to use and was less time consuming. The Wizard Genomic DNA purification kit was used for DNA extraction on the majority of samples and would be the most highly recommended. After extraction, DNA quantification was performed using the TKO 100 DNA Fluorometer (Hoefer Scientific Instruments) or the Ultrospec III spectrophotemeter (Pharmacia), after which all samples were stored at -20°C.

3.1. Phenol/Chloroform

This is standard methodology for purification of nucleic acids, using two organic solvents. Before starting this procedure, it is important to equilibrate the pH of phenol to 7.8-8.0, since it will partition into organic phases if too acid. This method is not recommended when DNA fragments larger than 30 KB are the target.

First, 500 µl of aqueous Lysing Buffer {5x multiplex buffer [1M Tris-HCl (pH 8.8), 1.0 M $MgCl_2$, 1.0 M $(NH_4)_2SO_4$, 0.1 M EDTA, 1.0 M 2-mercaptoethanol (BME)], 9% NP-40, 9% Tween-20, Proteinase K} was added to the cell pellet and allowed to digest for 1 hr. @ 65°C, followed by the addition of an equal volume of phenol-chloroform solution in a 1:1 ratio. Care was taken not to shear the DNA when the contents of the tube were completely mixed. The extracting suspension was centrifuged at 12,600 - 14,000 rpm for 2-5 minutes at room temperature in the microcentrifuge (Jouan®). After centrifugation, two distinct phases were present in the tube. The top aqueous phase containing DNA and buffer was pipetted out into a fresh microcentrifuge tube (Diamed

Lab Supplies, Inc.) and the entire process was repeated until no protein precipitate was visible at the interface of the organic and aqueous phases. For maximal DNA extraction, the organic phase was back extracted with equal volumes of TE buffer (pH 7.8). Any resulting aqueous product was added to the first aqueous phase. To remove traces of phenol, an equal volume of chloroform was added to the aqueous phase.

The DNA was recovered and concentrated by precipitation with 95% ethanol in a 2:1 ratio to the aqueous phase (Sambrook *et al.*, 1989). The tubes were then centrifuged at maximum speed at 0-4°C for 15 minutes. The supernatant was discarded and the DNA pellet was rinsed with 70% ethanol, which was carefully added down the side of the tube, so not to disturb the DNA pellet. As much ethanol as possible was removed without dislodging the pellet and any remaining alcohol was allowed to evaporate by air drying either at room temperature or at 37°C in an incubator. The DNA pellet was resuspended overnight in 50µl of TE buffer (pH 8.3) and DNA quantification was done using a spectrophotometer or fluorometer.

The spectrophotometer not only allowed for DNA quantification, the optical density at 260nm (OD_{260}), but also measured traces of protein present at 280nm (OD_{280}), thereby giving an indication of the purity of the extracted DNA. If the OD_{260}/OD_{280} ratio, did not fall between 1.8 -2.0, as per the manufacture's instructions, the sample was re-extracted since the OD reading was significantly less accurate if the DNA solution is contaminated with either protein or phenol. Although the fluorometer does not give an indication of the purity of the DNA, it is a more sensitive measure of DNA that requires less material for analysis.

3.2. Dialysis DNA Isolation Method

Cells were thawed and suspended in 5 ml of Cell Lysing Solution (CLS), consisting of 10mM Tris-HCl (pH 8.3), 140 mM NaCl, 3mM KCl, 0.35 M sucrose, 1 mM EDTA and 1% Triton X-100, in a 7 ml. capacity douncer tissue grinder (Wheaton) placed in an ice bath. While in the ice bath the suspension was slowly dounced 10 times with pestle B (loose pestle clearance) and then 10 times with pestle A (tight pestle clearance) in order to gradually break cell membranes and nuclear envelopes. The dounced solution was strained through a 100 μm-nylon mesh filter into a polypropylene tube and spun at 1100 g for 12 minutes at room temperature. The supernatant was discarded and with the tube turned upside down at a 45° angle the inside wall of the tube was dried with a cotton swab to prevent excess moisture from washing back onto the DNA pellet. The pellet was then washed with 3 ml of CLS, centrifuged for 5 minutes and dried with a cotton swab as above. To remove the DNA pellet from the wall of the tube, 55 μl of Dounce Buffer [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)], to which 20 μl/ml of RNAce It was added. For protein removal, 55 μl of prewarmed (50°C) proteinase K/SDS aqueous solution [0.1 mg. Proteinase K, 100 μl of 10% SDS, 100 μl of 0.5 M EDTA (pH 7.5)] was added to the solution. Gentle pipetting with a wide bore pipette tip resulted in a very viscous digestion solution which was incubated for 15-20 minutes at 50°C and then transferred with a wide bore pipette tip to a 0.45 μm Cell Culture Plate Insert filter (Millipore) floating in 35 ml of TE buffer. The digest was allowed to dialyze for 24-48 hours before

the genomic DNA was transferred from the filter to an microcentrifuge tube for fluorimetric DNA quantification (Rogers *et al.*, 1996).

3.3. Wizard Genomic DNA Purification Kit

This DNA extraction was adapted from the basic protocol included with the Wizard Genomic DNA Purification Kit (Promega). The mononuclear cell (MNC) pellet was resuspended with 200 µl of the cell lysing solution (CLS) supplied by the kit and vortexed until completely resuspended. A volume of 300 µl of nuclei lysing solution (NLC) was added and pipetted 5-6 times resulting in a very viscous lysate. If clumps of cells were still visible in the suspension then it was incubated at 37°C until fully suspended. After the addition of 1.5 µl of RNAse solution, the tube was inverted approximately 25 times to mix the solution and then incubated at 37°C for 15 minutes. After incubation, the samples were allowed to cool to room temperature before 150 µl of protein precipitation solution (PPS) was added. The nuclear lysate was vortexed for 10-20 seconds to mix and then centrifuged as above for 3 minutes. Care was taken not to disturb the brownish protein pellet when samples were removed from the centrifuge, as well as when the supernatant was transferred to a clean microcentrifuge tube containing 300 µl of 100% isopropanol at room temperature. A white thread-like strands of DNA formed when the sample was gently inverted several times. The DNA was centrifuged on maximum speed at room temperature for 15 minutes to create a small white pellet. The supernatant was quickly poured off the pellet by inverting the tube after which 300 µl of 70% ethanol was added and the DNA pellet was rinsed by gently inverting the tube. The sample was centrifuged at maximum speed for 30 minutes and with the tube inverted at a 45° angle the

supernatant was aspirated from the tube. Any remaining moisture was allowed to air dry and the samples were rehydrated in TE buffer (pH 8.3), either at room temperature over night or by incubating at 65°C for 1 hour. DNA quantification was done using the fluorometer or spectrophotometer as described previously.

4. V(D)J Recombination Assay

4.1. Nested Polymerase Chain Reaction (PCR) Amplification

Using previously published primers (Table III) and thermal protocols (Lipkowitz *et al.*, 1990), a nested PCR program was performed in a PCR System 9600 (Perkin Elmer Cetus) to amplify a hybrid fragment between TCR-Vγ and TCR-Jβ. The thermal protocol for each nested PCR can be found in Table IV. In order to determine potential PCR amplification of hybrid TCR fragments, both 100 and 1000 ng of DNA were used for each individual sample. The two DNA concentrations represent one magnitude difference in the dilution method previously reported (Lipkowitz *et al.*, 1990). At both the 1000 ng and 100 ng level of DNA per 50 µl reaction, individual sample results are the total of three independent PCR's performed at each DNA concentration.

Table III. Nested PCR primer sets to amplify hybrid TCR (Vγ - Jβ) as a result of aberrant V(D)J recombination activity. Oligonucleotide primer sequences were previously published by Lipkowitz *et al.* (1990).

Outside primers:	
Vγ-a	**5'-TAC ATC CAC TGG TAC CTA CAC CAG-3'**
Jβ-1a	**5'-TTC CCA GCA ACT GAT CAT TG-3'**
Inside primers	
Vγ-b	**5'-CTA GAA TTC CAG GGT TGT GTT GGA ATC AGG A-3'**
Jβ-1b	**5'-CCA GGA TCC CCC GAG TCA AGA-3'**

Confidence in the PCR methodology comes from several sources. Histopathological methods are estimated to detect 1 to 10% neoplastic cells relative to the

total cells in a biopsy sample, while molecular techniques are capable of detecting one neoplastic cell among 10^5 to 10^6 total cells (Crescenzi *et al.*, 1988; Sklar, 1993). PCR involving more than 20 replication cycles, has been reported to yield more than a million fold amplification (King and Stansfield, 1990). Zhang and Ehrlich (1994) reported very good detection of low copy number sequences using a semi-nested PCR approach to amplify a chromosome translocation t(14:18), between bcl-2 and J_H immunoglobulin gene sequence. They were able to detect a single DNA molecule from one mutant cell expressing this translocation in a background of normal DNA from 10^6 cells.

Table IV. Polymerase chain reaction (PCR) thermal protocol for amplification of aberrant V(D)J recombination event Vγ - Jβ.

Number of Cycles	Function	Temperature	Time
1	initial denaturation	95°C	2.5 min
25	cycle denaturation	95°C	30 seconds
	cycle annealing	61°C	30 seconds
	cycle extension	72°C	6 minutes
1	final extension	72°C	5 minutes
N/A	hold/ storage	4°C	indefinitely

In order to minimize amplification as a result of non-specific priming, minor modifications were made to the published protocol (Lipkowitz *et al.*, 1990). The modifications included increasing the annealing temperature in the PCR cycling program from 50°C to 61°C (Balinger, personal communication, 1996), omitting the use of gelatin (0.01% wt/vol) in the reaction, and substituting tetramethylammomiun chloride (TMAC) for dimethyl sulphoxide (DMSO) in the PCR buffer. DMSO (10%) is thought to decrease non-specific amplification by reducing inter- and intra-strand re-annealing and, therefore, improve PCR amplification. On the other hand, DMSO has also been found to inhibit

DNA synthesis by Taq polymerase by 50% compared to the use of TMAC (Hung *et al.*, 1990).

4.2. DNA Fragment Separation with Agarose Gel Electrophoresis and Imaging

To cover a wider range of fragment sizes, two DNA ladders, a 123bp (Gibco/BRL) and 1 Kb (Statagene) ladder, were loaded on all agarose gels as size markers. A volume of 10 µl DNA ladders and PCR product were loaded on 2% agarose gels containing (2 µg/ml) ethidium bromide. Gel electrophoresis was carried out for approximately 1.5-2 hours at 7-10 v/cm. The presence of DNA fragments were viewed, photographed and digitally stored using a Eagle Eye II Imager (Stratagene).

4.3. Statistical Analysis

Statistical analysis was used to compare the number of positive (present) or negative (absent) aberrant V(D)J recombination events in each of our four study groups. Independent multiple pair-wise comparisons were performed in order to determine the likelihood of our results having arisen by chance alone. For pair-wise comparisons, we considered the following three discrete variables: 1) the total number of individuals who exhibited/did not exhibit aberrant V(D)J recombination events in each group, 2) the total number of observed aberrant events in the total possible observable aberrant V(D)J events, not excluding possible clonal events, and 3) the total number of observed V(D)J events when considering their distribution across sixteen categories of PCR products, ranging from 250 - 2100 base pair.

Since the study groups were small, the two-tailed Fisher Exact Test, was chosen to compare numbers of individuals exhibiting aberrant V(D)J recombination events within

each group. The Chi square (χ^2) with Yate's correction, was used when comparing all possible aberrant V(D)J events. For comparing the distribution of independent aberrant V(D)J fragment sizes across the range of PCR products the Hypergeometric Test was used (Adams and Skopek, 1987). Both the Fisher Exact Test and the Chi square analysis were performed using Statistica (StatSoft) software.

To remove the element of error due to multiple comparisons in our analysis, the Bonferroni procedure was applied to the 0.05 α-level (Wassertheil-Smoller, 1995). As we presented 6 pair-wise comparisons within each group, the *p* values were only considered significant if the number fell below the Bonferroni adjusted α-level value of 0.0083 for all three analyses.

5. Microsatellite Assay

Seven microsatellite markers were investigated for germline alterations in all our AT family members (Table V). Six markers are simple dinucleotide repeat sequences, while the *APOC3* marker has a complex hypervariable repeat.

Table V. Description of seven microsatellite markers used to investigate germline alterations in Ataxia Telangiectasia family members (after da Cruz *et al.*, 1997).

MI Marker	Marker Location	(Repeat Sequence) # of repeats	Fragment Size (bp)
D11S35	11q22	$(GT)_{17}$	152 - 162
APOC3	11q23-24	$(C_a(T_b))_c$	327 - 359
p53	17p13.1	$(CA)_n$	103 - 135
nm23-H1	17q22	$(CA)_n$	94 - 104
Ank1	8p11-21.1	$(AC)_n$	107 - 113
D8S135	8p12-21.3	$(GT)_n$	152 - 162
D6S105	6p	$(CA)_{23}$	116 - 138

a= a range from 1 to 3; b= from 1 to 10; c= from 50 to 54.

All seven microsatellite oligonucleotide primers were chosen because they had been implicated in human breast cancers in other studies in addition 2 of the markers (*APOC3* and D11S135) were reported to flank the region of the AT gene in chromosome 11q22-23 (Litt *et al.*,1991).

5.1. Touch-down PCR Methodology

In order to run one PCR for all primers under the same thermal profile, a touch-down thermal protocol (da Cruz, 1997) was used to optimize annealing temperatures for each primer set (Table VI). This protocol increases primer specificity and therefore reduce non-specific amplification. The protocol involved a reduction in the annealing temperature within the cycling program by 1°C per cycle from a temperature of 65°C in the first cycle down to 55°C in the tenth cycle. An additional 20 cycles at 55°C preceeded a final extension temperature of 72°C for 5 minutes.

Table VI. The touch-down thermal protocol giving optimal annealing temperatures in the presence of multiple primers (after da Cruz *et al.*, 1997).

Number of Cycles	Function	Temperature	Time
1	initial denaturation	94°C	5 min
30 in total: (1 cycle/ temp)- 20	cycle denaturation	94°C	1 min
	touchdown annealing	(65 ⇒ 56°C) -55 °C	1 min
	cycle extension	72°C	1 min
1	final extension	72°C	5 min
N/A	hold	4°C	indefinitely

5.2 DNA Fragment Separation using Polyacrylamide Gels and Imaging

For microsatellite fragment separation, 20 µl of PCR products were run on a vertical 7% non-denaturing polyacrylamide gel (7% deionized polyacrylamide, 2x TAE, 10% ammonium persulfate, 0.001% N,N,N',N'- tetramethylethlenediamine) for 5.5 - 6.5 hours at about 7 v/cm. A mix of two DNA ladders, 25 bp and 123 bp in a ratio of 2:1, was

used to estimate fragment size. The gel was removed from the glass plate and allowed to soak in 2 μg/ml ethidium bromide for 10-15 minutes. The Eagle Eye II Imager (Stratagene) was used to view and store the microsatellite marker fragment images.

5.3 Assessment of Microsatellite Instability (MIN)

Samples from both biological parents were necessary in order to classify results as fully informative. The lack of either parental sample meant the offspring were designated as only semi-informative. Distant relationships of the AT relatives were not informative in the detection of novel alleles in the AT children. Nevertheless, all samples were assayed to determine whether PCR products for these markers would fall into expected size range. Because only the maternal grandfather (UN06) was available, siblings of F1H1 can only be considered semi-informative. Likewise, UN01 is semi-informative. Therefore the investigation of family polymorphism's could not be considered fully informative.

An AT patient would have been considered positive for microsatellite instability due to germline alterations if they displayed a novel allele not ascribed to either of the parents. The presence of a single allele for any marker in the offspring would not necessarily indicate allele loss, but may indicate a homozygous state if the parents both displayed this allele. The PCR protocol was repeated for all samples that did not produce a PCR product to insure PCR failure was not considered as a negative result.

CHAPTER III. Results

Genomic DNA was extracted from peripheral mononuclear cells of AT homozygotes, AT Heterozygotes, AT Relatives and Controls. Both the V(D)J recombination assay and the determination of germline mutation of microsatellite markers were used as an indicator to differentiate between these groups.

1. The V(D)J Recombination Assay

We investigated potential differences in the frequency of hybrid TCR occurrences due to aberrant V(D)J recombination activity. Results of the aberrant hybrid TCR in the study groups can be found in Table VII and Table VIII. The distribution of aberrant events ranged from 225 - 2100 bp within all four groups (raw data - see Appendix A). However, there was a wide variation of fragment sizes within individual samples, altogether comprising 16 different size catergories (Table VIII).

There were no significant differences between the groups when comparing the number of individuals within the groups that expressed these aberrant events (Table IX) and the distribution of the PCR products sizes (Table XI). No difference was found for both PCR reactions using 100 ng and 1000 ng of DNA per reaction, respectively.

Table VII. Presence (+ve) of hybrid TCR events as observed within the four study groups, both at the individual expressing the event as well as observed events in the total possible events.

Presence of V(D)J Events	DNA (ng/rxn)	Control	AT Patients	AT Heterozygotes	AT Relatives
+ve Individuals (Group)	100	8/12 (66%)	3/4 (75%)	4/5 (80%)	4/6 (66%)
	1000	21/25 (84%)	1/4 (25%)	4/5 (80%)	5/6 (83%)
Observed (Total Possible)	100	18/572 (3.1%)	32/192 (16.6%)	15/240 (6.3%)	22/288 (7.6%)
	1000	86/1200 (7.1%)	9/192 (4.7%)	13/240 (5.4%)	37/288 (12.8%)

Table VIII. Frequency distribution of aberrant hybrid TCR fragments within 16 size categories observed in study populations at both 100 and 1000 ng DNA per PCR reaction.

Fragment size (bp)	Control		AT Patients		AT Heterozygotes		AT Relatives	
	100	1000	100	1000	100	1000	100	1000
225	n/o*	1	n/o	n/o	n/o	1	n/o	n/o
250	4	14	3	1	n/o	2	2	5
275	n/o	1	1	n/o	n/o	1	n/o	n/o
400	n/o	1	1	1	n/o	1	1	3
500	n/o	2	n/o	1	n/o	n/o	n/o	2
600	n/o	2	n/o	n/o	n/o	n/o	2	2
750	4	16	2	1	3	2	4	4
900	1	4	2	n/o	n/o	n/o	2	2
1000	5	14	3	1	3	3	1	3
1100	n/o	2	n/o	n/o	n/o	n/o	n/o	n/o
1250	1	7	1	n/o	2	1	2	3
1300	n/o	n/o	n/o	n/o	1	n/o	n/o	n/o
1350	n/o	n/o	n/o	n/o	1	n/o	n/o	n/o
1500	n/o	2	1	n/o	n/o	n/o	1	1
1600	1	1	1	n/o	1	n/o	1	1
2100	n/o	n/o	1	n/o	1	n/o	n/o	n/o
Total	16	67	16	5	12	11	16	26
n	12	25	4	4	5	5	6	6

* Not observed.

Table IX. *p* values obtained after Fisher Exact Test for 6 two-way comparisons between the four study groups. The groups were considered significantly different if the numbers of individuals expressing aberrant hybrid TCR within each group resulted in a $p \leq 0.00833$ (Bonferroni adjusted α-level).

Groups[1]	Control	AT Patients	AT Heterozygotes	AT Relatives
Control		1.0	1.0	1.0
AT Patients	0.0339		1.0	1.0
AT Heterozygotes	0.9781	0.2063		1.0
AT Relatives	0.9624	0.1905	1.0	

[1] Cells on the lower left half of the table represent values from PCR performed using 1000 ng of DNA per reaction, while the cells on the upper right half of the table are from PCR performed using 100 ng of DNA per reaction.

When assessing the presence of aberrant hybrid TCR, we counted all independent events observed in three repeat experiments. Some of the groups differed significantly at both the 100 ng and 1000 ng level of DNA (Table X). At a level of 1000 ng of DNA per reaction, only AT relatives were significantly different from Controls (p=0.0025), AT heterozygotes (p=0.0013), and AT patients (p=0.005). At the level of 100 ng of DNA per

reaction both AT relatives (p=0.005) and AT patients (p=0.000) were different from Controls. The AT patients were again found to be significantly different from the AT Relatives (p=0.0035) and as well as from the AT heterozygotes (p=0.001).

Table X. *p* values obtained after Chi-square Test with Yate's correction for 6 two-way comparisons between the four study groups. The groups were considered significantly different if the presence of aberrant hybrid TCR in the total possible observations resulted in a $p \leq 0.00833$ (Bonferroni adjusted α-level).

Groups[1]	Control		AT Patients		AT Heterozygotes		AT Relatives	
	χ^2	*p* value	χ^2	*p* value	χ^2	*p* value	χ^2	*p* value
Control			**41.2**	**0**	3.5	0.616	**7.87**	**0.005**
AT Patients	1.23	0.27			**10.9**	**0.001**	**8.52**	**0.0035**
AT Heterozygotes	0.7	0.40	0.01	0.9			0.20	0.65
AT Relatives	**9.15**	**0.0025**	**7.94**	**0.005**	**10.4**	**0.0013**		

[1] Cells on the lower left half of the table represent values from PCR performed using 1000 ng of DNA per reaction, while the cells on the upper right half of the table are from PCR performed using 100 ng of DNA per reaction.

Table XI. *p* values obtained after the Hypergeometric Test for 6 two-way comparisons between the four study groups. The distribution of independent aberrant hybrid TCR across the range of PCR product sizes was considered significantly different if the test resulted in a $p \leq 0.00833$ (Bonferroni adjusted α-level).

Groups[1]	Control	AT Patients	AT Heterozygotes	AT Relatives
Control		0.9065	0.4085	0.5070
AT Patients	0.4925		0.6965	0.8930
AT Heterozygotes	0.6950	0.9300		0.4320
AT Relatives	0.6715	1.0	0.6500	

[1] Cells on the lower left half of the table represent values from PCR performed using 1000 ng of DNA per reaction, while the cells on the upper right half of the table are from PCR performed using 100 ng of DNA per reaction.

2. Microsatellite Instability (MIN) Assay

The determination of germline alterations in the AT family members was performed by using seven microsatellite markers. Both, Family #1 and Family #3 can be considered as fully informative for germline change since samples were obtained from the biological parents and their children. **Error! Reference source not found.**(a and b)

depicts typical patterns of PCR products of six of the microsatellite markers used for Families #1 and #3. In addition, Figure 6 displays the D11S35 marker in all three AT families. In both Family #1 and Family #3, none of the children (F1P1, F3P1 and F3P2) expressed novel alleles for any of the seven markers assayed. Although F2P1 showed an allele which was not observed in his biological mother (F2H1) this could not be considered a truly novel allele, since it may have been donated by his father, who declined the invitation to donate blood for this study.

The AT relatives could not be considered fully informative since only one biological parent was represented. However, it was observed that the four siblings of F1H1 (UN02, UN03, UN04, and UN05) and their father (UN06) expressed seemingly identical fragment sizes for markers *ANK1* (Figure 7), *APOC3*, and D8S135 (figures not shown). For markers *p53*, *nm23-H1*, D6S105 and D11S35, these samples expressed alleles of varying sizes. However, none of the observed variations fell outside the expected size ranges for their respective markers. UN01, the daughter of UN05, always expressed at least one allele that was present in the mother (figures not shown).

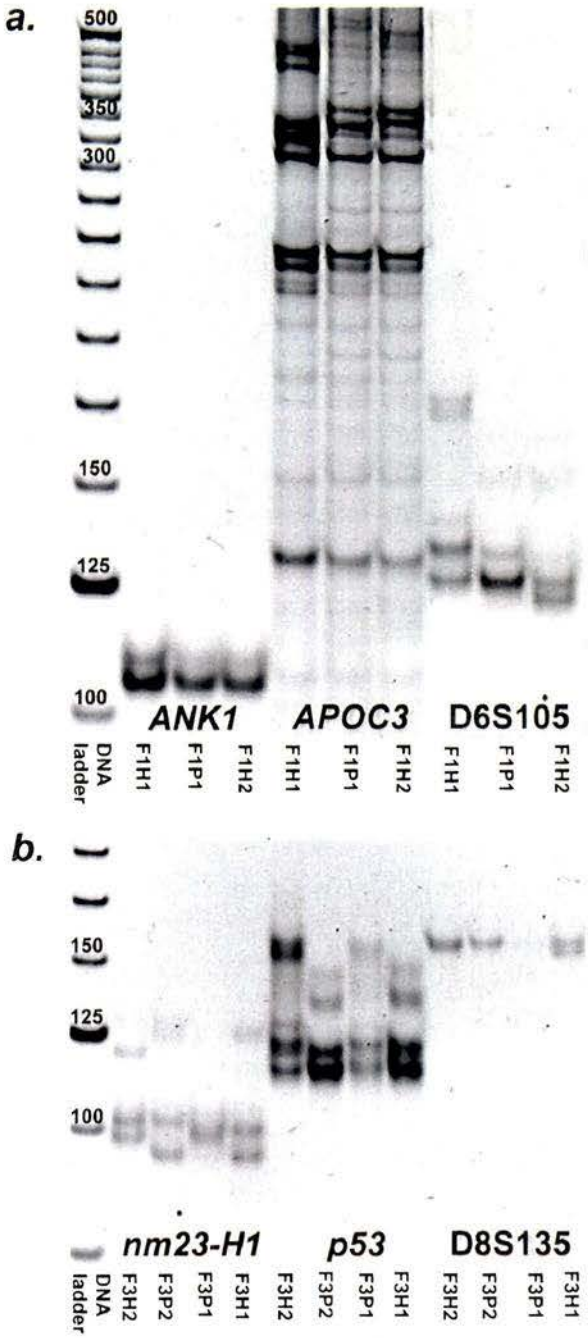

Figure 5. Six microsatellite markers for a) AT Family #1: mother (F1H1), son (F1P1) and father (F1H2); b) AT Family #3: father (F3H2), son (F3P2), daughter (F3P1) and mother (F3H1). The expected fragment sizes are (bp): *ANK1* (107-113), *APOC3* (327-359), D6S105 (116-138), D8S135 (152-162), *p53* (103-135) and *nm23-H1* (94-104). F3P1 shows no band for D8S135, however, reamplification displayed an allele in the expected size range.

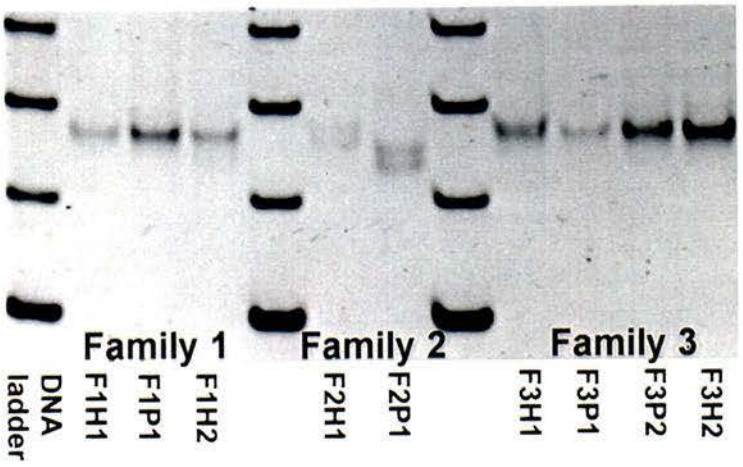

Figure 6. Microsatellite marker D11S35 for 3 Ataxia Telangiectasia families. Family #1 and Family #2 are represented by both parents and their offspring, while Family #2 is represented by the biological mother and her son. The expected fragment size for D11S35 is 152-162 bp.

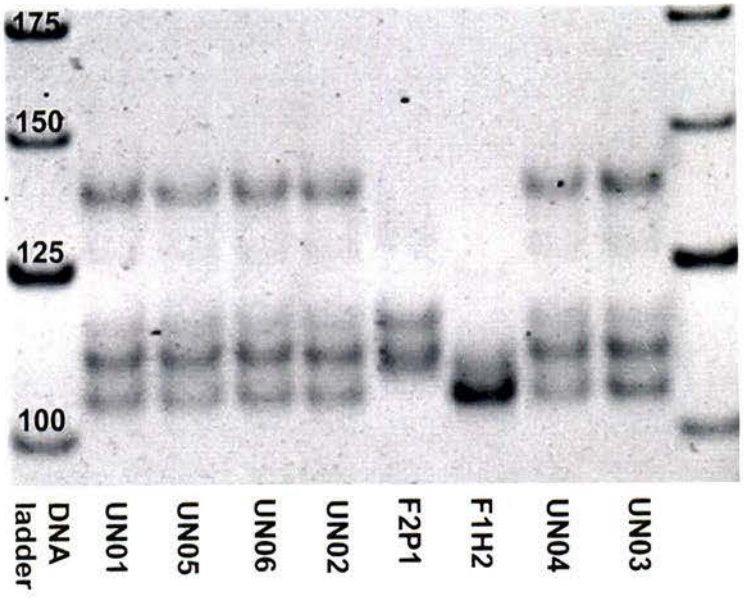

Figure 7. Microsatellite marker *ANK1*, with an expected size range of 107-113 bp, for AT relatives (extended family members of F1H1).

CHAPTER IV. Discussion and Conclusions

There is extensive evidence that individuals heterozygous for the Ataxia Telangiectasia gene are at increased risk for breast cancer development (Swift *et al.*, 1976; Swift *et al.*, 1987; Pippard *et al.*, 1988; Swift *et al.*, 1990; Morell *et al.*, 1990; Swift *et al.*, 1991; Easton, 1994; Athma *et al.*, 1996). Therefore, the development of strategies to distinguish AT carrier status in the general population would be a useful tool in clinical trials and follow-up studies despite the social and ethical issues that must be considered. Although this dissertation will not elaborate on these issues, we must acknowledged there are many open-ended questions. As with the BRAC1 and BRCA2 testing capabilities, the public demand for "that new breast cancer test" will likely surpass the laboratory and clinical resources. Once the system is overwhelmed, who sets the criteria of whom should be tested and when? Although funding has increased for breast cancer research in recent years, funding agencies generally fail to acknowledge the psychosocial and ethical issues that are part and parcel of genetic testing research. As a society, are we ready to deal with the answers to the breast cancer susceptibility gene question? What will the institutions of healthcare and corporate insurance do with the information? Finally, if prophylactic mastectomy is currently the only putative form of primary prevention (Baron and Borgen, 1997), why will women want to know?

The identification of AT heterozygotes would allow for familial, lifestyle and medical management options, including increased monitoring and early detection of malignancies, especially breast cancers. Early detection of oncogenic processes is still the

strongest correlating factor for increased survival rate. The research and medical communities must remain sensitive to the impact genetic testing has on the decision making processes of individuals involved. Being found at increased risk for breast cancer may cause individuals to consider more serious health management protocols as early health care options, including radical prophylactic mastectomy. Yet, as Athma and colleagues (1996) have suggested, the increased risk for AT heterozygotes may not be associated with early age onset of breast cancer, as with the hereditary breast cancer susceptibility genes BRCA1 and BRCA2. The perception of increased risk for an individual may out weight the scientific understanding of risk. Furthermore, the high frequency of unique mutations may slow our awareness of the risks associated with particular mutations of *ATM*. If future studies distinguish differences in modes of action between breast cancer susceptibility genes and even the location of mutations within these genes, raising public awareness to these differences will be important.

Additionally, as the AT defect is characterized by an increased sensitivity to ionizing radiation, knowing the individual AT status would impact on health care decisions, such as recommendations for decreased occupational and medical exposures, including mammography. In the event of cancer, this increased sensitivity could also have implications on clinical treatment in terms of radiation therapy. For instance, if loss of heterozygosity (LOH) was found responsible for tumour tissue development in an AT heterozygote, all the tumour cells would be AT homozygous and hypersensitive to cell killing by ionizing radiation.

The *ATM* is a large gene with a high frequency of unique mutations that currently prevents the use of mutation specific assays for general population screening (Gilad *et al.*,

1996b). Here, we have investigated the use of the V(D)J recombination assay and the microsatellite instability assay (MIN) as two potential endpoints to discriminate the AT status among our four study populations, namely, AT homozygotes, AT heterozygotes, AT relatives and Controls.

1. V(D)J Recombination

Assuming that increased frequency of aberrant hybrid TCR, as previously published (Lipkowitz *et al.*, 1990), represents a real measurement of an aberrant process which is exacerbated in some way by the AT defect, then AT homozygotes should always display the highest measures of aberrant hybrid TCR. On the other hand, AT heterozygous individuals might be expected to be intermediate to AT patients and controls populations. With an unknown number of AT carriers in the group of AT relatives, aberrant hybrid TCR results might be expected to be more varied. However, the results would be expected to range between controls and AT heterozygotes.

Here we report no significant differences between the study groups from the three criteria of investigation for aberrant V(D)J recombination events. For two of the statistical criteria (i.e., the number of individuals expressing aberrant V(D)J events in each group and the frequency distribution of the PCR fragment sizes) no differences between the groups were found in using either 100 or 1000 ng of DNA per PCR reaction. However, there were differences in the percentage of individuals in which events were seen if comparing the groups at 1000 ng and 100 ng. Using the Lipkowitz *et al.* (1990) rationale, one might assume that in reactions using 1000 ng of DNA a random aberrant event may be present in any sample type and be successfully amplified. By reducing the amount of DNA, only groups that had increased levels of the aberrant hybrid TCR would amplify a PCR

product. Lipkowitz and colleagues (1990) reported that the intensity and detection level of aberrant hybrid TCR was consistently greater in AT samples than in normal samples. In our study, this was not always the case. At 100 ng of DNA the AT homozygotes did have 75% (3/4) of individuals expressing V(D)J events while the other groups ranged from 66 - 80% (Table VII). However, when 1000 ng of DNA was used, only 25% (1/4) of the AT homozygous presented recombination events while the other groups ranged from 80 - 84%. Similarly, the number of aberrant hybrid TCR observed in the AT homozygous group when using 100 ng of DNA was 16.6% (32/192), while the other groups ranged from 3.1 - 7.6% (Table VII). However, at 1000 ng of DNA the AT homozygotes exhibited the lowest frequency of aberrant hybrid TCR, comprising only 4.7% (9/192). AT heterozygotes the second lowest frequency at 5.4% (13/240), followed by the Control group with 7.1% (86/1200). The AT relatives exhibited the highest frequency, accounting for 12.8% (37/288).

It has been suggested that all V(D)J recombination elements may not be amplified equally at higher DNA concentrations, with shorter PCR products being amplified more efficiently in the presence multiple competitive targets (Lipkowitz *et al.*, 1990). Conversely, they reported that larger PCR products amplified more successfully at lower DNA concentrations. This rationale does not appear to explain the sporadic pattern of individual differences generated in our data. However, we do report on one AT homozygote (F3P1) who amplified the largest fragment size (2100 bp) in one of three independent PCR reactions using 100 ng of DNA. In addition, F3P1 failed to amplify any PCR products in three independent assays at 1000 ng of DNA per reaction. In the case of one heterozygote (F2H1), a 2100 bp fragment was detected in only one of three

independent assays using 100 ng of DNA per reaction. PCR of F2H1 using 1000 ng DNA per reaction amplified fragments from 225 bp - 1000 bp but not 2100 bp. Regardless of fragment size, the frequency of chimeric TCR at the lower dilution should not appear higher than the frequency at the higher concentration of DNA, especially since DNA for PCR using 100 ng/reaction was obtained by dilution from the 1000 ng concentration.

If our previous assumptions are valid, the AT heterozygotes should have exhibited less hybrid TCR events than AT homozygotes, which was not the case. Here, AT heterozygotes did not show a reduction in the number of aberrant events from 1000 ng (13 in 240 possible events) to 100 ng (15 in 240 possible events). Our results suggest that increased frequency of hybrid TCR are not an accurate measure of an aberrant V(D)J recombination process which may be exacerbated in AT patients. Neither is it a useful endpoint to discriminate AT heterozygotes from the population at large. A kinder assessment would acknowledge that there are possible confounding factors, including lifestyle, dietary questions, and potential PCR artifacts, which are perhaps contributing to the outcome of our results.

The importance of lifestyle factors has been demonstrated in an assessment of genotoxic events in pesticide-exposed Italian floriculturists (Scarpato *et al.*, 1996). When measuring induction of sister chromatid exchanges (SCE), structural chromosomal aberrations (CA), and micronuclei (MN), no statistical difference was found between exposed and non-exposed individuals. However, when considering heavy smoking as a lifestyle factor, there was a significant increase in SCE and CA levels, 17% and 54% respectively (Scarpato *et al.*, 1996).

We performed preliminary work with preseasonal blood samples collected from Indo-Canadian agricultural workers involved in the berry picking industry of the Fraser Valley, British Columbia, Canada (data not shown). The group of agricultural workers was not significantly different from our other study populations for neither the number of individuals expressing aberrant events nor for the frequency distribution of these aberrant hybrid events. Statistical analysis of the number of events in all possible events for the group of agricultural workers showed a significant difference from controls and AT relatives. However, it must be stressed that the difference was due to a lesser number of aberrant events observed in the agricultural workers when compared to all other groups. Although few of these workers declared themselves as vegetarians, in general, there was decreased consumption of red meats, coffee and soft drinks in their diets.

Other researchers have reported increased aberrant hybrid TCR and other chromosome aberrations in pesticide-exposed workers as a transient state and the increase is reduced and eliminated over time, suggesting the role of T-cell turnover (Lipkowitz *et al.*, 1992; Carbonell *et al.*, 1995). We currently know of no clearance mechanisms that would specifically target the removal of hybrid TCR from the circulation. However, T-lymphocyte half-life has been reported using both cytogenetic endpoints and the *hprt* gene, ranging from 0.6 - 3 years for the former and 2.1 years for the latter (da Cruz *et al.*, 1996). Current estimates of T-cell turnover do not support the rapid decrease in elevated frequencies of hybrid TCR to control levels after 4-6 months of non-exposure. A proportion of T-cells carrying hybrid TCR should persist in the peripheral pool even in the absence of seasonal pesticide exposure.

Differences in metabolic activation of environmental agents and detoxification capacities have been suggested in the literature as an explanation for individual variations in genotoxic measures (Wild *et al.*, 1995; Scarpato *et al.*, 1997, Josephy, personal communication, 1997). An investigation of polymorphisms in two forms of the human detoxification protein glutathione S-transferase, GSTM1 and GSTT1, found genetically determined variations in the ability to metabolize hazardous compounds in pesticide-exposed greenhouse workers (Scarpato *et al.*, 1997). Although pesticide exposure was not associated with elevated frequencies of CA, smokers lacking a functional GSTM1 gene showed statistically significant increases in CA levels (2.75±0.31) as compared to GSTM1 positive smokers (1.56±0.32, p=0.026).

Genetic polymorphisms in genes that determine the rate of metabolic inactivation of environmental carcinogen exposure could hold some answers for a sub-set of seemingly unrelated breast cancer incidences (Josephy, personal communication 1997). Although individuals may not have mutations in known "breast cancer genes", individual genetic variations in metabolic activation genes may increase susceptibility for breast cancer. As reported above, an individual's inability to metabolize environmental carcinogens (a slow metabolizer) increases exposure time to the toxic agent(s) and it's metabolites, putting the individual at increased risk for the development of tumours. Josephy's laboratory is currently investigating applicability of the individual susceptibility model in breast cancer development. It is hypothesized that polymorphisms in the N-acetyltransferase (NAT) gene provides individual variation in the rate of metabolism (fast versus slow metabolizers) of environmental exposures to aromatic amines. Furthermore, their preliminary data shows mammary tissue specificity, supporting the result of peroxidases present as an integral part

of lactation and breast tissue function. Individual metabolic activation and detoxification capacities have a major impact on chromosomal integrity. In assays that measure chromosomal damage or aberrant chromosomal processes, individual metabolic variations could obscure research outcomes, for example, the ability to assess AT gene status in the general population.

It must also be mentioned that we questioned the validity of our initial results with the V(D)J assay. When PCR amplification products were observed in the majority of individuals in all groups, we attempted to tighten the stringency of the assay by increasing the annealing temperature from 50°C to 61°C in an effort to reduce what we presumed to be non-specific amplification. Additionally, we performed a serial dilution and used a DNA fingerprinting method (data not shown) to more closely model the published assay. Although, we did observe some of the anomalies as mentioned by Lipkowitz *et al.* (1990), such as amplification of PCR products at lower dilution's when not found at higher dilution's, these findings were not consistently reproducible and did not discriminate the study groups. Other researchers invested substantial time trying to optimize this protocol but the project was abandoned and no publications on the preliminary work have resulted to date (Balinger, personal communication). Taken together, the inability to reproduce the published data by ourselves and other researchers prevents our recommending the V(D)J recombination assay as a potential tool for screening AT-carrier status in the general population.

5.2. Microsatellite Instability

Somatic alterations of microsatellite markers have proven a useful tool in determining genomic instability in cancer research, helping to elucidate possible

mechanisms of gene involvement and mutator phenotype in tumourogenesis (Hall *et al.*, 1992; Cropp *et al.*, 1993; Winqvist *et al.*, 1993; Patel *et al.*, 1994; Carter *et al.*, 1994; Hampton *et al.*, 1994; Negrini *et al.*, 1995; Winqvist *et al.*, 1995). In this study, we examined the potential use of microsatellite loci to investigate inherited germline alterations, which could contribute to mutator phenotypes and, therefore, to tumour predisposition, especially breast cancer.

If cancer incidences in the AT homozygotes is reported 100-fold increased over the general population (Athma *et al.*, 1996), it would be useful to know when these individuals deviate from the rest of the population. We know from studies investigating somatic microsatellite alterations between normal and tumour tissue that microsatellite instability represents a late step in tumour progression. However, colon cancer research has suggested germline MIN in DNA repair genes (Thrash-Bingham *et al*, 1995). To our knowledge there have been no reports on germline microsatellite alteration in AT homozygotes. Of the three informative AT patients in our study (F1P1, F3P1, and F3P2), microsatellite germline alterations were not detected. Therefore, we suggest that general elevated genomic instability, as expressed by MIN, does not appear to be indicative of AT status.

The general consistency of the inherited alleles in this study appears compatible with literature reports that alleles are inherited in a stable manner from one generation to the next (Jeffreys *et al.*, 1985; Darvasi and Kerem, 1995). From our results, it does not appear that the AT phenotype affects the individual's ability to inherit microsatellite alleles in a stable fashion. However, the lack of novel alleles in these few AT patients as

compared to their biological parents in no way eliminates the possibility that the instability is present in one of the adults and inherited by the children in a stable fashion.

Our interest is in using microsatellite markers as a screening tool for genetic instability that may be a predisposing factor for cancers. The fact that different samples resulted in similar fragment sizes does not mean they do not hold a mutation. To confirm the presence or absence of a mutation, sequencing analysis would need to be performed. This would increase the time, labour and equipment requirements and therefore decrease it's usefulness as a screening tool. Also, the investigation of germline microsatellite alterations requires larger sample collections, of extended family members. From our experience with sample collections we know this is a difficult task and would decrease its usefulness as a screening tools but would prove feasible in families where an index individual had been identified.

Although AT heterozygotes are at increased risk for breast cancer (Swift *et al.*, 1991; Athma *et al.*, 1996), the pattern of risk associated with this gene are different from those normally associated with cancer susceptibility genes. The AT gene locus has not been linked to premenopausal bilateral breast cancer (Cortessis *et al.*, 1993), and the greatest risk for breast cancer in AT heterozygous individuals occurs after 60 years of age (Athma *et al.*, 1996). The increased risk for breast cancer in AT heterozygous after 60 years of age which could be due, in part, to increased medical radiation exposures. Since breast cancer is generally considered a slow growing cancer, taking 20 years or more to become a detectable tumour, this is not an unlikely hypothesis. From this thinking, the ability to identify AT heterozygotes might alter current monitoring strategies and recommendations for medical radiation exposures, including mammograms.

Five of the women in our study, one of whom is an obligate AT heterozygote, are related siblings who qualify or will soon qualify for mammography screening programs. It would be of interest to follow these women to investigate future potential molecular alterations. Since all these women are currently cancer free and show no microsatellite alterations, our study could provide a baseline monitoring against which future assays are compared.

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Glossary[3]

appendicular cerebellar tremours. Involuntary trembling movement relating to the appendages or limbs, relating to the cerebellum, the large posterior portion of the brain.

cerebellar ataxia. An inability to perform coordinated muscular movements, relating to the cerebellum.

Choreoathetosis. Abnormal movements of body of combined choreic (spasmodic) and athetoid (convulsive) pattern of the limbs and facial muscles

dysarthia. Disturbance of articulation due to emotional stress or to paralysis, in-coordination, or spasticity of the muscles used for speech.

dystonia. A state of abnormal (either hypo- or hyper-) tonicity in any of the tissues.

Extrapyramidal. Other than the pyramidal tract in the motor cortex.

hemochromatosis. A disorder of iron metabolism.

Huntington's chorea. Hereditary disorder consisting of spasmodic, involuntary movements of the limbs or facial muscles.

hypotonic facies. A defect in facial muscular tone or tension resulting in a slack expression or appearance in the face.

idiopathic scoliosis. Lateral curvature of the spine lacking clear pathogenesis. Usually consisting of two curves, the original abnormal curve and a compensatory curve in the opposite direction.

microcephaly. Abnormal smallness of the head often seen in mental retardation; it is congenital.

muscular hypotonia. The slow initiation and performance of voluntary movement.

oculocutaneous telangiectasia. The marked dilation of small blood vessels at the conjunctiva of the eye.

oncocytoma. An adenoma composed of eosinophilic epithelial cells, especially of the salivary or parathyroid glands. These are benign tumours

progeric. Relating to premature senility occurring in childhood.

senile keratosis. Dry, harsh skin of the aged.

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[3] Terms as defined by Taber's Cyclopedic Medical Dictionary and/or the Illustrated Stedman's Medical Dictionary.

Appendix A.

1.a. Aberrant hybrid TCR products for AT homozygotes (F#P#) and AT heterozygotes (F#H#) from three independent PCR reactions using 1000 ng DNA/reaction.

Sample	F1P1			F2P1			F3P1			F3P2			F1H1			F1H2			F2H1			F3H1			F3H2					
Size (bp)	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
225																				+										
250										+	+	+	+						+	+										
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1.b. Aberrant hybrid TCR products for AT Relatives from three independent PCR reactions using 1000 ng DNA/reaction.

Sample	UN01			UN02			UN03			UN04			UN05			UN06														
Size (bp)	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
225																														
250	+		+		+	+	+				+	+						+												
275																														
400			+			+						+																		
500								+			+	+																		
600			+									+																		
750	+		+	+	+	+	+	+								+	+	+												
900			+															+												
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1250	+				+												+													
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1.c. Aberrant hybrid TCR products for Control Group from three independent PCR reactions using 1000 ng DNA/reaction.

Sample	BC96			BC97			BC98			BC107			BC112			BHC113			BC115			BC116			BC117			BC119		
Size (bp)	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
225																														
250	+		+						+			+					+					+	+				+		+	+
275																														
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750	+	+				+	+	+	+								+					+	+			+			+	
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1.c. (cont.) Aberrant hybrid TCR products for Control Group from three independent PCR reactions using 1000 ng DNA/reaction.

Sample	BC122			BC123			BC124			BC125			BC127			BC130			BC139			GN01			GN02			CN01		
Size (bp)	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
225																														+
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1.c. (cont.) Aberrant hybrid TCR products for Control Group from three independent PCR reactions using 1000 ng DNA/reaction.

Sample	CN02			CN03			CN04			L2A			L39-1																	
Size (bp)	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
225																														
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2.a. Aberrant hybrid TCR products for AT homozygotes (F#P#) and AT heterozygotes (F#H#) from three independent PCR reactions using 100 ng DNA/reaction

Sample	F1P1			F2P1			F3P1			F3P2			F1H1			F1H2			F2H1			F3H1			F3H2					
Size (bp)	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
225																														
250						+	+	+	+	+	+	+																		
275												+																		
400										+	+	+																		
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750							+	+	+	+	+	+	+							+	+				+					
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1000						+	+	+	+		+	+			+							+	+	+			+			
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1600							+																			+				
2100							+												+											

2. b. Aberrant hybrid TCR products for AT Relatives from three independent PCR reactions using 100 ng DNA/reaction.

Sample	UN01			UN02			UN03			UN04			UN05			UN06														
Size (bp)	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
225																														
250			+							+		+																		
275																														
400										+		+																		
500																														
600			+							+																				
750	+		+			+				+	+	+				+		+												
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1250						+												+												
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1500				+																										
1600																	+													
2100																														

2.c. Aberrant hybrid TCR products for Control Group from three independent PCR reactions using 100 ng DNA/reaction.

Sample	BC96			BC98			BC113			BC116			BC117			BC122			BC123			BC127			BC139			CN01		
Size (bp)	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
225																														
250	+		+	+																					+					
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2.c. (cont.) Aberrant hybrid TCR products for Control Group from three independent PCR reactions using 100 ng DNA/reaction.

Sample	CN02			CN04																										
Size (bp)	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
225																														
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Bebb G, Steele PP, Warrington PJ, Moffat JA and Glickman BW (1997): Caffeine does not potentiate γ-radiation induced DNA damage in Ataxia Telangiectasia lymphoblastoid cells. *Mutation Research* (submitted).

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Microsatellite Instability and Aberrant V(D)J Recombination in T-lymphocytes of Ataxia Telangiectasia Affected Individuals, Obligate Heterozygous and Unaffected Family Members.

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August 18, 1997