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as a Risk Factor for Radiation-Associated Breast Cancer

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FOREWORD

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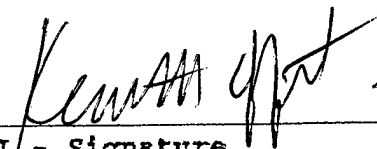

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I. INTRODUCTION

Ataxia-telangiectasia (AT) is an autosomal recessive disorder characterized by neurologic abnormalities (cerebellar degeneration, ataxia, and progressive mental retardation), dilation of blood vessels (oculocutaneous telangiectasia), immune deficiencies, and premature aging.¹ AT homozygotes also suffer from an approximately 100-fold increase in cancer incidence, with leukemias and lymphomas being particularly common.²⁻⁴ A single gene, *ATM*, appears to be mutated in the majority of AT families studied to date.⁵ The exact function of the *ATM* gene product is unknown, but the coding sequence bears similarity to yeast genes that serve cell cycle checkpoint and DNA repair functions.⁶⁻⁹ Evidence suggests that *ATM* functions as a central component of the cellular response to DNA damage.¹⁰

AT heterozygotes do not manifest the multisystem abnormalities characteristic of the homozygous state. However, these individuals may also be prone to develop malignancies. In a large prospective follow-up study of 161 AT families, the risk of all cancers in female heterozygotes was 3.5 times that of non-carriers.¹¹ In particular, heterozygous females appeared to have at least a 5.1 times excess risk of breast cancer. Smaller, retrospective studies have also indicated that these individuals may suffer from an increased risk of cancer in general and female breast cancer in particular.^{4,12,13} The issue of AT heterozygote cancer susceptibility is not trivial. Although AT is a rare disease, the population frequency of AT heterozygosity is estimated to be 1.4%, and Swift has speculated that up to 8% of all breast cancers in the United States may occur in women who are carriers of an abnormal AT allele.^{12,14} The true prevalence of *ATM* mutation among unselected women with breast cancer is probably considerably lower than this figure. Using an exon-scanning PCR single-strand conformation polymorphism (PCR-SSCP) assay, Vorechovsky *et al* were unable to detect any *ATM* mutations in 38 unselected primary breast cancer cases.¹⁵ A recent analysis of 401 women with early-onset breast cancer demonstrated a mutation frequency of only 0.5%.³⁵ Even accepting the limitations of this method and the possible existence of intronic or regulatory mutations, it is unlikely *ATM* mutations are present in a significant fraction of unselected women with breast cancer. However, because of design limitations, the available studies are unable to exclude a significant relative risk associated with the inheritance of an *ATM* mutation, particularly if a second insult is required to unveil the inherited susceptibility..

It remains possible that women who carry a mutant *ATM* allele are predisposed to breast cancer after exposure to a particular environmental factor, specifically radiation. Swift and his coworkers have suggested that diagnostic, therapeutic, or occupational exposure to radiation may predispose heterozygotes to the development of cancer.¹¹ This observation is biologically consistent with reports that cultured fibroblasts from AT heterozygotes suffer from an *in vitro* defect in the cellular response to radiation damage.^{16,17} Unfortunately, the available studies suffer from significant methodological limitations and the conclusions are by no means universally accepted.¹⁸ A creative approach is required to prove or refute Swift's hypothesis.

If women carrying a single mutant *ATM* allele are indeed susceptible to the genotoxic effects of radiation, one would expect to see an excess number of *AT* heterozygotes among women with radiation-induced breast cancer. The identification of such women is generally problematic due to difficulties in the quantitation of radiation exposure and in the establishment of a cause-effect relationship between that exposure and the subsequent development of malignancy. However, one well-defined group that does appear to be prone to develop radiation-associated breast cancer is women receiving therapeutic irradiation for Hodgkin's disease (HD). Several large studies have determined that women receiving radiotherapy for HD have a relative breast cancer risk of 1.3-2.2 when compared to controls.¹⁹⁻²⁶ The latency period is quite long, and the risk appears to be most significant after 15 years of follow-up.^{20,21,24} Women who receive their radiotherapy before the age of 30 appear to be more prone to develop radiation-associated breast cancer, and those treated during adolescence have the greatest risk of all.^{20,21,27} In a series collected at Stanford University, women receiving radiotherapy between the ages of 10 and 19 years were 39 times more likely than controls to develop breast cancer in their third decade.²¹ Interestingly, this relative risk correlates with an absolute risk of approximately 1.6%,²⁸ which is similar to the projected frequency of heterozygosity for *ATM* mutation in the general population.

Yahalom *et al* had previously identified 37 patients treated at MSKCC for breast cancer occurring after radiotherapy for HD.²⁹ We propose to study this cohort for the presence of *ATM* mutations and to perform a case-control to define the risk of breast cancer associated with such mutations. This novel approach will test the hypothesis that *ATM* mutations predispose women to breast cancer after radiation exposure. The approach is particularly innovative because it utilizes a cohort with defined radiation-associated cancer, and is not hindered by the difficulties inherent in studies that attempt to retrospectively attribute cancer to radiation exposure.

II. BODY

A. Protocol Submission and IRB Approval

After DAMD17-97-1-7147 was awarded, the full protocol was submitted to the Memorial Sloan-Kettering Institutional Review Board for approval. Provisional approval had been granted in November 1996, and the relevant consent form had been forwarded to the Breast Cancer Research Program as required. Formal approval for the study was obtained on June 24, 1997 and the study was assigned local protocol number 97-81. Patient ascertainment and recruitment began immediately.

B. Subject Ascertainment/Recruitment

The first cohort of women to be approached regarding study participation were the women reported in Dr. Yahalom's paper of 1992. Of these 37 women, 3 were known to be deceased and permission to contact was refused by the primary physician of 1. Of the

remaining women, 3 were lost to follow-up with no recent address, 3 declined to participate, and 7 have deferred deciding whether or not they wish to participate. These latter women have been contacted several times and, as yet, have not agreed to donate a sample for testing.

Of the original cohort, 20 women have undergone genetic counseling, provided informed consent, and donated a blood sample. An additional 13 women with breast cancer after treatment for Hodgkin's disease have consented and donated after having been identified by clinicians of the Radiation Oncology and Breast Cancer Medicine services. The processing of these specimens will be described below in the Methods section.

All participants have undergone complete pretest genetic counseling. In the context of that counseling, information has been gathered regarding the clinical features of the breast cancer and Hodgkin's disease diagnoses, as well as the personal risk factors for breast cancer. Individuals with an apparent inherited predisposition to breast cancer have been identified and counseled as to their testing options (e.g. *BRCA1* or *BRCA2*), although such testing has not and will not be provided as part of this study. A clinical and administrative ("tracking") database has been created, and data entry initiated.

Although the major thrust of sample ascertainment during the first year of the project has been on individuals with breast cancer after treatment for Hodgkin's disease, identification and recruitment of suitable controls has also begun. To date, 5 controls have provided consent and a blood sample. In addition, the parents of a child with ataxia-telangiectasia have donated samples to serve as positive controls for the mutation detection techniques being developed.

C. Laboratory Methods

1. cDNA Preparation

To facilitate mutation detection, lymphoblastoid cell lines have been established from peripheral blood lymphocytes for 30 cases by Epstein-Barr-virus transformation.³⁶ Total RNA was then isolated from the lymphoblastoid cell lines using the Ultraspec RNA isolation system (Biotecx Laboratories, INC., Houston, TX) according to manufacturer's instructions. RNA pellets were resuspended in 100µl of DEPC treated water. The total RNA was reverse transcribed with random hexamers using the Superscript cDNA Preamplification Kit (Life Technologies) to generate cDNA. Reverse transcriptions were carried out in a volume of 20µl containing 1-3 µg of RNA, 100 ng Random Hexamers, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 0.5 mM dNTP, 10 mM DTT, and 200U Superscript II Reverse Transcriptase. The reaction tubes were incubated at 42°C for 60 min and then 70°C for 15 min to terminate the reaction. The

product was treated with 2U E.coli Rnase H and incubated at 37°C for 20 min. The cDNA was then diluted with 20µl sterile water and stored at -20°C. A 2.5µl aliquot of cDNA was used as a subsequent PCR template.

2. Protein Truncation Testing (PTT) Analysis

PTT presents several advantages when compared to other mutation screening methods. Large gene segments of 2-3Kb can be analyzed at the same time in one reaction and mutations that lead to truncated protein products are efficiently detected.^{37,38} Since more than 70% of the mutations in the ATM gene lead to truncated proteins, we chose PTT for initial screening of patient-derived cDNAs for mutations in the 9.2Kb fragment.

In order to generate products suitable for PTT analysis, the entire coding region of the 9.3 Kb of ATM transcript and the adjacent untranslated regions have been divided into 7 overlapping regions (a-g). Forward primers have been designed that include a T7 promoter sequence for the initiation of transcription by T7 RNA polymerase and an ATG sequence for initiation of translation. Primers being used for the PTT analysis and their respective optimal annealing temperatures are shown in Appendix 1.

PCR of each region was carried out in a volume of 50µl containing 1.5 mM MgCl₂, 20 mM Tris-HCl (pH 8.0), 50 mM KCl, 200 µM dNTP mix, forward and reverse primers at 10 pM, and 3.75U of Platinum Taq DNA Polymerase (Life Technologies). PCR of each region consists of an initial denaturation of 3 min at 94°C, 35-40 cycles of 1 min at 94°C, 1 min at 50-56°C, 3 min at 72°C and a final extension step for 10 min at 72°C. The PCR amplification conditions for all the 7 regions have been optimized (Appendix 1) for further PTT and REF (Restriction Endonuclease Fingerprinting) analysis.

To perform the actual PTT analysis, a 13 µl reaction mix containing 5µl of RT-PCR product, 6.25µl of TnT rabbit reticulocyte lysate (Promega), 0.5µl of [35S] methionine (1000 Ci/mmol, NEN Life Science), 0.25 µl TnTT7 RNA polymerase, 0.25 µl of methionine-deficient amino acid mixture, and 0.25 µl of RNAsin (40u/µl) will be incubated at 30°C for 1.5 hr. Following the PTT, 5ul of product will be electrophoresed on a 15-20% SDS-polyacrylamide gels. Following the electrophoresis, the gel will be dried and subjected to autoradiography overnight.

Any PCR fragment producing a truncated protein, along with the wild-type product will be subjected to cycling sequencing (Amersham Life Science). Two µl of PCR product will be used for sequencing. The sequencing reaction will be carried out in a volume of 7µl containing 2-2.5

pg of DNA, 2 pM sequencing primer, 0.5 unit polymerase, 0.5 μ l [33 P]ddNTP, and 2ul of dGTP mixture. The samples were denatured at 95°C for 3 min, followed by 45 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min. After amplification, 3 μ l of stop solution (95% formamide, 10 mM NaOH, and 0.25% bromophenol blue) will be added. The mixture will be electrophoresed on a 6% sequencing gel in 1X TBE buffer (0.09 M Tris-borate, 0.002 M EDTA). Gels will be fixed, dried, and exposed to film at room temperature for 12 h or more, after which the sequence will be interpreted in the usual fashion.

3. Restriction Endonuclease fingerprinting (REF)

The REF method is an attractive method in terms of labor and costs, especially when large transcripts are analyzed. In this method, the size of the fragment analyzed in a single lane may be 10 times that of SSCP (Single Strand Confirmation Polymorphism). Since most sequence alterations are expected to shift more than one band on REF gel, the efficiency of detection should exceed that of SSCP. The ability of this method to detect deletions and insertions as well as base substitutions in homozygotes and heterozygotes which are otherwise missed by PTT has been documented.^{31-33,39}

The REF protocol of Liu and Sommer will be followed, with slight modifications.³¹ Amplified DNA (100ng) will be digested separately with 4-6 restriction endonucleases in the presence of 0.2U shrimp alkaline phosphatase (U.S. Biochemicals, Cleveland, OH).³⁹ Following heat inactivation at 65°C for 5 min. the digestion products will be pooled, denatured at 96°C for 10 min, and immediately chilled on ice. Ten ng of this fragment mixture will be labeled in the presence of 6 μ ci of gamma[33 P]ATP and 1U of T4 polynucleotide kinase(New England Biolabs, Beverly, MA) for 45 min at 37°C. Twenty μ l of stop solution containing 95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol, and 10 mM NaOH will be added; the samples boiled for 3 min and quick chilled on ice. The fragments will be separated by electrophoresis on 5.6% polyacrylamide gels in 50 mM Tris-borate, pH 8.3, 1 mM EDTA at constant power of 12W for 3h at room temperature; The gels will be dried and subjected to autoradiography for 1-2 days at -70°C.

D. Discussion

The project is proceeding according to the timetable described in the statement of work. Cases have been identified, counseled, and have provided consent. Samples have been collected and cryopreserved, and lymphoblastoid cell lines have been created. The

mutation detection portion of the project is also underway, with the current focus on developing protein-truncation assay to screen for mutations leading to premature termination of the protein product and a Restriction Endonuclease Fingerprinting (REF) assay to detect missense mutations. To date, there are no results from mutation analysis, and thus there are no data bearing upon the central study question.

In the upcoming year, attention will be paid to expanding the ascertainment. The study coordinators have been unable to identify any additional women at Memorial Sloan-Kettering with breast cancer occurring after a diagnosis of Hodgkin's Disease. We are continue advertising efforts during presentations at national meetings and during lectures. In addition, we have asked the University of Pennsylvania and Stanford University to join us in this effort. The participation of these institutions will increase the pool of potential participants and should easily allow us to reach our goal of 50 patients and 50 controls.

III. CONCLUSIONS

The project is proceeding according to the Statement of Work. The local ascertainment of cases in nearly complete, and additional sites have been invited to participate to expand the number of cases available for study. Identification and recruitment of controls is underway. The laboratory mutation detection methodology is being developed and should be operational within the next 6 months.

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Appendix 1. PTT^a Primers being used for Mutation Screening of the ATM Gene

NAME OF THE PRIMER	NUCLEOTIDE SEQUENCE	TM ^b C ^b	FRAGMENT SIZE (bp)	REGION AMPLIFIED
ATMe	Forward(T7) ^c -GAAGTTGAGAAATTAAAGC	50.0°	1316	76-1392
	Reverse AATGCAACTTCCGTAAGGC			
ATMf	Forward(T7)-GCAGATATCTGT	55.0°	1769	1048-2817
	Reverse GTAGGTTCTAGCGTGCTAGA			
ATMg	Forward(T7)-AATGACATTGCAGATATT	55.0°	1655	2437-4092
	Reverse TCAGTGCTCTGACTGGCACT			
ATMa	Forward(T7)-ACGTTACATGAGCCAG	50.0°	1387	4048-5435
	Reverse TCCAAATGTCATGATTTTCAC			
ATMb	Forward (T7)-CTGGCCTATCTACAGC	55.0°	1247	5282-6529
	Reverse CAACCTGCTAAGTGGGAT			
ATMc	Forward (T7)-CAGTGGGACCAATTGC	55.0°	1534	6322-7856
	Reverse TTCTGACCATCTGAGGTCTCC			
ATMd	Forward (T7)-GATCACCCCATCACA	55.0°	1521	7651-9172
	Reverse TCACACCCCAAGCTTCCATC			

a Protein Truncating Test

b Annealing Temperature

c Promoter sequence and Translation start codon were added to all the forward primers at their 5' end
GGATCCTAATACGACTCACTATAGGAACAGACCATG.