Rare Variants of *ATM* and Risk for Hodgkin's Disease and Radiation-associated Breast Cancers[1]

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ABSTRACT

Purpose: **In this study, we first sought to evaluate whether individuals heterozygous for *ATM* mutations may have an increased susceptibility to radiation-induced breast cancer (BC) after treatment for Hodgkin's disease (HD). We next sought to determine the frequency of *ATM* variants in patients with Hodgkin's lymphoma, regardless of coexisting BC, compared with healthy volunteers.**

Experimental Design: **Full sequence analysis of *ATM* was performed on cDNA from peripheral blood lymphocytes from 37 cases of BC after therapeutic radiation therapy for HD and 27 comparison cases with HD and no BC treated during the same time period. The frequency of *ATM* variants was analyzed in the total group of 64 cases of HD and compared to allele frequencies in 128 ethnically matched controls from the same geographical region.**

Results: **No protein-truncating *ATM* mutations were observed in cases with HD with or without BC. Missense mutations were more frequent in the cohort with HD compared with patients with BC following HD ($P = 0.02$). The median time from HD to the development of BC was 18 years in patients with *ATM* variants compared with 16 years in those with no *ATM* variants ($P = 0.04$). Multiple *ATM* variants, including one homozygous mutation, were observed in 9 HD cases.**

Conclusions: **Heterozygous protein-truncating or missense mutations of *ATM* were not associated with increased radiation-associated risk of BC after HD. The observation of multiple germ-line mutations and a homozygote suggests that rare *ATM* variants may constitute cancer-susceptibility alleles in a subset of cases.**

INTRODUCTION

AT[4] is an autosomal recessive disorder characterized by neurological abnormalities, dilation of blood vessels, immune deficiencies, and premature aging (1). AT homozygotes also are at ~100-fold increased risk for cancers, predominantly leukemias and lymphomas (2–4). The *ATM* gene sequence bears similarity to yeast genes that mediate cell cycle checkpoints and DNA repair (5–9). *ATM* phosphorylates *BRCA1* and other targets activating a process of DNA repair through homologous recombination in cooperation with *BRCA2*, *mRAD51*, and other molecules (10).

Cells derived from AT patients are hypersensitive to ionizing radiation (11), and there is evidence that *ATM* haploinsufficiency also results in increased sensitivity to sublethal doses of ionizing radiation in mice (12). Epidemiological studies have noted an increased risk for BC in mothers of AT patients (4, 13, 14). The risk for BC after radiation therapy to the chest may be increased as much as 30-fold in young patients with HD (15). Taken together, these findings have raised the hypothesis that diagnostic radiation exposure may pose a BC risk factor for ~1% of the population that carry mutations of *ATM*. Prior studies have generally not confirmed an increase in proportion of heterozygotes for *ATM*-truncating mutations among patients with a family history of BC (16), a personal history of BC (17, 18), bilateral disease (19), or BC after radiation therapy for HD (20, 21). In the current study of patients with BC following HD and patients with HD treated with radiotherapy, we sought to compare the frequencies of protein truncating, as well as missense mutations of *ATM*, recognizing the potential for detection of *ATM* variants of unknown functional significance. We therefore compared the frequencies of *ATM* variants observed in these groups to healthy controls to elucidate a possible pathogenetic role of these variants in Hodgkin's lymphoma.

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[4] The abbreviations used are: AT, ataxia-telangiectasia; HD, Hodgkin's disease; MSKCC, Memorial Sloan-Kettering Cancer Center; BC, breast cancer; RT-PCR, reverse transcription-PCR.

Table 1 Clinical characteristics of the cohorts

	HD/BC (n = 37)	HD (n = 27)
Median age of HD (range)	23 yr (12–42)	25 yr (11–37)
Median age of BC (range)	39 yr (28–61)	
Median follow-up		17 yr (12–35)
Median interval between HD & BC (range)	18 yr (2–35)	

PATIENTS AND METHODS

Patient Population

Sixty-four cases of HD were identified for this study. This included 37 patients with histories of both BC and HD, identified from an original cohort of women seen at MSKCC (22) between 1956 and 1989 or from the University of Pennsylvania (one case). Of 37 cases from the MSKCC database, 36 consented to participation in this study. A comparison group of 27 patients were identified from the files of patients undergoing radiation therapy for HD at MSKCC from 1965 to 1992. None of the 27 patients in the comparison group had developed BC. Of the 27 patients in the comparison group, 22 were matched to HD/BC cases to ±6 years of both age at diagnosis and year of diagnosis. Seventy-five percent of the patients in the HD/BC group had stage I/II disease compared with 76% in the HD comparison group. Mean dose of radiation was 3900 cGy in the HD/BC group compared with 3600 cGy in the comparison group. Both paternal and maternal country of origin were assessed for all patients. Of the 37 HD/BC cases, 13 (35%) were of eastern European Ashkenazi ancestry, compared with 8 of 27 (29%) of the HD comparison group. Smaller proportions of both groups were of mixed Italian, English, German, Greek or other European ancestry; none were of African ancestry. All cases and comparison groups in this study were restricted to female gender. Other clinical characteristics of these groups are summarized in Table 1.

All patients had undergone genetic counseling and provided informed consent before donation of a blood sample. In addition, the parents of a child with AT provided consent and donated samples to serve as positive controls for the development of the mutation detection techniques.

A control group unaffected by cancer was derived from the prenatal genetic testing program at New York University. The mean age of the controls at time of genetic testing was 34 years of age. For each case of HD, the country of origin of the parents was ascertained. Each case was matched with two control individuals whose parents descended from the same country of origin as the parents of the cases. A total of 128 individuals (256 chromosomes) from the control group were genotyped for all mutations. In the analyses of the variant D1853N, additional samples were available from ethnically matched individuals. Therefore, the number of chromosomes available for the analysis of D1853N was 298 chromosomes.

Laboratory Methods

Protein Truncation Assay. Lymphoblastoid cell lines were established from peripheral blood lymphocytes by EBV transformation. Protein truncation testing analysis was performed as described previously (23, 24). RNA isolated from the lymphoblastoid cell lines was reverse transcribed with random hexamers using the Superscript cDNA Preamplification Kit (Life Technologies, Inc., Carlsbad, CA) to generate cDNA. The entire coding region of the *ATM* transcript, composed of 63 exons covering 9.2 kb, was divided into 7 overlapping regions. RT-PCR was used to produce transcription templates for protein truncation testing.

Sequence Analysis. Isolation of total RNA from peripheral blood was performed by Tri-Reagent BD (Molecular Research Center, Cincinnati, OH) according to the manufacturer's protocol. ATM transcripts were scanned for mutations in cDNA derived from RT-PCR spanning 9355 bp of the entire ATM open-reading frame and part of the untranslated 3′ and 5′ regions. The details of this protocol are available upon request. Briefly, this involved PCR of two partly overlapping fragments: RA (4964 bp) and RB (5062 bp). These fragments were amplified with the primers ATMin and AR for RA and 2xx and ATMR for RB in a final volume of 50 μl, including 1 μl of the RT product, 1 μl of 1 mg/ml BSA (BioLabs, Beverly, MA), 1 μl of 25 pM of each primer, 5 μl of 10× buffer 3 (Boehringer, Germany), 2.5 μl of 10 mM deoxynucleotide triphosphate (Boehringer), 0.75 μl of Expand Long Template (Boehringer, Germany), and 0.2 μl of Anti-*Taq* (Chimerx, Milwaukee, WI). The RA and RB fragments were cleaned using the Qiagen PCR purification kit, and 200 ng of each fragment were sequenced with Big Dyes PE ABI Prism 377 (primers available upon request). For analysis of the chromatograms, Sequencher software 3.0 (Gene Codes Corp., Ann Arbor, MI) was used. The PCR fragments were also examined by agarose gel electrophoresis to identify large deletions or insertions. All genotypes obtained by RT-PCR and direct sequence analysis were confirmed using genomic DNA derived from patient samples. Sequence variants identified in CDNA were verified by sequencing of genomic DNA from lymphocytes. The region that included the variant was amplified by PCR and the two strands were sequenced.

Detection of Specific Mutations. To detect individual mutations identified by sequencing, a rapid method of mutation detection was used. An amplification-created restriction enzyme site strategy was devised for 12 of the mutations detected by sequence analysis. In seven cases, naturally occurring restriction sites were identified, and for five of the mutations, PCR conditions were modified to create restriction enzyme sites near the suspected mutant sequence and can be provided upon request. For the 5557G→A mutation (D1853N), it was necessary to devise a strategy of denaturing high-performance liquid chromatography to screen for this mutation (25). All cases and controls shown to be carrying mutations by amplification-created restriction enzyme site or denaturing high-performance liquid chromatography were confirmed by sequencing.

Statistical Methods

The end point was time to BC, measured as the time elapsed (in years) between the age at diagnosis of HD to the date of diagnosis of BC (for the 37 patients with HD and BC) or the last follow-up time (for the 27 patients with HD only). The presence of an *ATM* variant was recorded as a binary variable ("yes" if the individual had a missense mutation, "no" if the individual had no detected mutation). Kaplan-Meier estimates of

Table 2 *ATM* Variants seen in 64 patients with HD and normal controls

Mutation					
Nucleotide change	Amino acid change (codon)	HD (n = 27)	HD/BC (n = 37)	Total cases (mutations/no. of chromosomes)	Controls (mutations/ chromosomes)
1541 G/A	G514D1			1/128	0/256
1810 C/T	P604S	2		2/128	7/256
2119 T/C	S707P	2	1	3/128	2/256
2289 T/A	F763L		1	1/128	2/256
2572 T/C	F858L		1	1/128	2/256
3161 C/G	P1054R	2	1	3/128	7/256
4258 C/T	L1420F	1	2	3/128	2/256
4388 T/G	F1463C	2		2/128	4/256
5089 A/G	T1696A	1		1/128	0/256
5557 G/A	D1853N	8	6	14/128	37/298
5558 A/T	D1853V	1(#)[a]	2	4/128	4/256
6235 G/A	V2079I	1		1/128	4/256
6919 C/T	L2307F	2		2/128	3/256

[a] #, mutation observed in homozygous state; note: some variants were observed together in single patients.

time to BC among patients with and without an *ATM* mutation were compared using the Wilcoxon test. Proportions of cases and controls with specific or multiple variants were compared by Fisher's exact test. All *P*s are two-sided.

RESULTS

No protein-truncating mutations were identified in the 64 cases of HD, including the 37 patients with both HD and BC and 27 patients with HD treated by radiation therapy but who did not develop BC. Sequence analysis, however, identified 13 missense mutations among the 64 cases. Of these, three variants have not previously been reported, and 10 variants are represented in prior publications or online databases.[5] New variants not previously reported include F763L, F1463C, and T1696A. Seven variants were observed in 11 of 37 HD/BC cases, whereas 11 variants were observed in 16 of 27 age-matched HD cases without BC.

The median time to develop BC was 18 years, and the median follow-up (based on the 27 patients with HD who did not develop BC) was 17 years. Clinical characteristics of the patients are given in Table 1, and the individual *ATM* variants are listed in Table 2. As shown in Fig. 1, the median time to BC after HD was 18 years for patients with an *ATM* variants *versus* 16 years for those without any variant (P = 0.04; Wilcoxon test). Of the 37 patients who developed BC after HD, an *ATM* variant was seen in 11 (29.7%) patients, compared with 16 of 27 (59.3%) of those with HD only (P = 0.02).

The frequency of individual *ATM* variants was analyzed in samples of chromosomes from healthy individuals (see Table 2). The median age of HD in cases was 24 years, and the median age of healthy controls at time of genotyping was 34 years. There was also no difference in frequency of any individual variant among the 64 patients with HD with or without BC and the healthy controls matched for ethnic origin. The occurrence of multiple *ATM* variants was analyzed in 128 samples derived

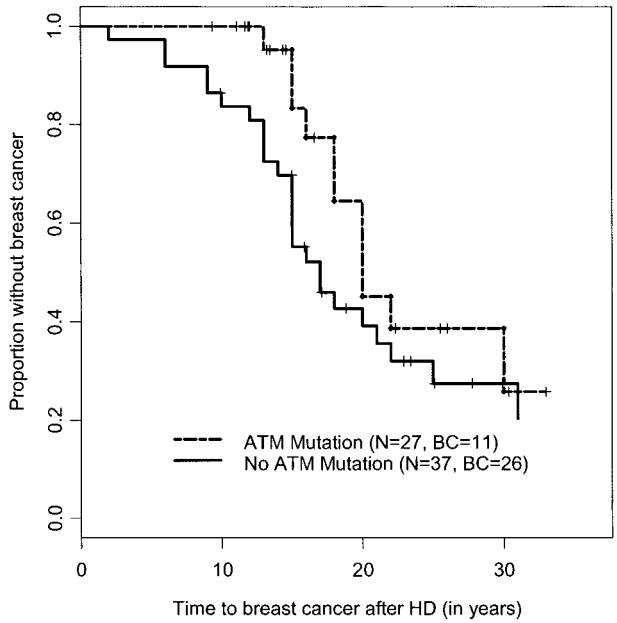

Fig. 1 Time-to-event analysis of BC after HD among patients with and without an *ATM* missense mutation.

from healthy individuals of similar ethnic and geographical origin as the cases. More than one missense mutation was observed in 9 of 64 (14.1%) cases, compared with 7 of 128 (5.6%) controls (P = 0.054). In 2 of the cases, there were 3 variants. In one of the HD cases, the patient was homozygous for the variant D1853V. Of the 7 healthy controls with more than one *ATM* variant, none carried more than two variants, and none were homozygous for any of the mutations.

No particular ancestry of parental origin was significantly more common in the group with *ATM* variants compared with the group without these variants. Of the 8 patients with multiple *ATM* variants listed in Table 3, 3 (38%) were of Eastern European Jewish background, compared with 21 of 64 (33%) cases overall. Of significance, however, the 2 cases and 4 healthy

[5] Internet address: www.vmresearch/org/ATMut-t.htm.

Table 3 HD cases and controls carrying multiple *ATM* variants

Case no.	Phenotype	Mutations
Cases with multiple variants		
25	HD	L1420F, T1696A
34	HD	D1853V, D1853V (HOMOZYGOTE)
39	HD	S707P, P1054R
48	HD	S707P, D1853N
52[a]	HD	P604S, F1463C, D1853N
54	HD/BC	F858L, P1054R
60	HD/BC	D1853N, S707P
68[a]	HD	P604S, F1463C, L2307F
70[a]	HD/BC	D1853N, D1853V
Controls with multiple variants		
2011, 1246		F858L/P1054R
1141[a], 3230[a], 594[a], 828[a]		P604S/F1463C
793		D1853N/P1054R

[a] Ashkenazi ancestry.

controls in whom P604S and F1463C were coincident were all of Eastern European (Ashkenazi) Jewish origin, suggesting a common origin of this haplotype. Another putative haplotype was F858L/P1054R, observed in 1 case and 2 controls of similar northern European ancestry. It was possible to obtain a DNA sample from both parents of the case; genotyping confirmed that both variants segregated on the same chromosome. Excluding the P604S/F1463C and F858L/P1054R associations, the six remaining combinations of multiple *ATM* variants shown in Table 3 were not observed in the healthy control group. D1853N/P1054R was observed in a single control but in none of the cases. Thus, excluding the 3 cases and 6 controls with either the P604S/F1463C or F858L/P1054R putative haplotypes, 6 of 60 (10%) cases and 1 of 122 (0.8%) healthy controls demonstrated multiple *ATM* variants ($P < 0.01$). Moreover, in both of the cases carrying the P604S/F1463C, there was a third variant present as well: the D1853N in 1 case (no. 52) and the L2307F in another (no. 68). A combination of these three *ATM* variants was not observed in the controls.

DISCUSSION

This study includes the largest number of radiation-associated BC cases after HD reported to date. In addition, the full sequence analysis approach of this study differs from prior analyses based on protein truncation-based methods of mutational screening (16, 17, 20, 26, 27). Assuming a heterozygote frequency of ~1% in the general population, the absence of detection of truncating mutations of *ATM* in the HD/BC group in this study excludes a large effect of protein-truncating *ATM* mutations as radiation-associated BC risk factors. Combined with prior studies, ~100 women with BC following HD have now been studied with no *ATM*-truncating mutations seen. Although prior epidemiological studies have shown that risk for BC after radiation therapy for HD may be as high as 31-fold, (15) the risk associated with *ATM* mutations has been projected to be in the range of 5-fold (14). Although these studies effectively exclude a relative risk of 20–30 for BC associated with *ATM*-truncating mutations in this radiation-exposed group, the possibility remains that that less substantially elevated risks for radiation-associated BC may be associated with protein-truncating *ATM* mutations.

In contrast to what would have been expected based on assumptions of radiosensitivity of *ATM* heterozygous cell lines, this study documents a slightly greater median time to develop BC in the cohort of cases with rare *ATM* mutations compared with those who received the same radiation and did not have *ATM* mutations. In addition, the frequency of *ATM* variants was significantly greater in the HD cohort compared with the HD/BC cohort. The modest separation of the time-to-event curves (Fig. 1) may have resulted from the greater number of *ATM* missense mutation carriers with short follow-up, however, the median follow-up time for the cohort without BC was very close to the median time to develop BC (16 *versus* 17 years). Although continued follow-up is necessary and the power of the time-to-event comparisons is limited by the small sample size, these results do not support the hypothesis that rare variants of *ATM* are associated with a shorter time to radiation-associated BC.

Potential biases in this study include a prevalence or survivor bias because cases of HD may not have represented incident cases. This bias would not have affected selection of the HD/BC cases because these were all incident cases. However, the comparison group of HD cases were selected among HD survivors who had not developed BC, and this group would not have included patients who died because of HD or complications of treatment. If *ATM* variants were associated with a decreased survival, such a bias would have resulted in underrepresentation of these mutations in the HD comparison group. Instead, however, *ATM* variants appeared more frequently in the comparison group. Another potential source of bias results from the age of the healthy control group. Although comparable with the cases with respect to age at diagnosis of BC, the cases developed HD, on average, 10 years before the age at which the healthy controls were genotyped. Thus, those controls who had developed and died of HD may have been underrepresented in the control group. However, given the very low incidence of HD and the excellent outcome of treatment, effects of this bias would be minimal.

Ten of 13 *ATM* variants observed in this report have been described in prior studies. G514D was observed in 1 of 93 individuals with no known genetic disease in a recent survey; this individual was 1 of 18 of African ancestry included in the series (28). P604S, which creates a new glycosylation site, was seen in 1 of 192 BC cases in a prior series (29). S707P was observed in an early-onset BC case described by Izatt *et al.* (30) This variant occurs in a conserved region in the murine *ATM* (31), which shows 91% amino acid similarity to human *ATM* protein. In addition, we predict that the S707P abolishes a putative glycosylation site. This variant, observed in excess in unselected BC cases compared with controls (29, 32), was observed in two lymphoma patients with no history of BC in the current series. F858L was initially described as a deleterious mutation and was associated with partial inactivation [65% expression of atm protein in leukemia cases (30, 33)]. Some studies have documented the F858L allele at polymorphic (>1%) frequencies in both BC cases and controls (28–30, 32–34), but other

series have documented this allele in cases but not in normal controls (35). P1054R was observed in 1 of 38 BC patients and 5 of 224 (2.3%) chromosomes of European Caucasians (18), as well as in AT families, where it was classified as a variant seen at allele frequency of 0.07 (28). P1054R was also documented at polymorphic frequencies in normal controls in two recent series (29, 32). L1420F was observed in 53 of 1380 (3.8%) of BC cases and 24 of 905 (2.6%) of controls in combined data from four series (18, 26, 29, 32). F1463C was felt to be a deleterious mutation based on its discovery in obligate heterozygous parents of homozygous affected children (36). D1853V was previously reported at an allele frequency of 0.03 (28) and had been described in 9 of 723 (1.2%) controls in three recent series (28, 29, 32). V2079I was observed in 1 of 38 breast tumors and 3 of 224 (1.3%) chromosomes from European Caucasians (18). Finally, L2307F was observed in 1 of 142 BC cases and 0 of 81 controls in a recent series (32).

Considered individually, there was no significant excess in prevalence of *ATM* variants in patients with HD, regardless of coincidence of BC, compared with healthy controls. Thus, there was no evidence supporting a predisposition to Hodgkin's lymphoma associated with individual *ATM* variants in heterozygous state. An increased prevalence of germline *ATM* variants has been reported in patients with T-cell prolymphocytic leukemia (37). However, a report of 16 patients with T-cell prolymphocytic leukemia indicated that the common *ATM* mutations in these patients were of somatic rather than germ-line origin (38). An increased frequency of *ATM* missense mutations has been documented in recent series of BC cases compared with controls (29, 32); the current series represents the first such analysis of patients with lymphoma. One patient in this series was homozygous for the rare variant D1853V, a finding that is not consistent with random assortment of these alleles. The D1853V homozygote was observed in 2 of 128 chromosomes from the cases, whereas D1853V heterozygotes were observed in 4 of 268 chromosomes (1.5%) from the controls. The expected frequency of homozygotes would therefore be 0.0002 in comparison to 0.016 observed. This corresponds to an odds ratio (estimate of risk for HD) of 72.2 (95% confidence interval = 3.4–529) for this rare variant. Expanded analysis of the mode of inheritance of combinations of rare *ATM* variants will be necessary to derive a more precise estimation of risk appropriate to the mode of transmission of these alleles in populations.

An unexpected finding of this study was the high number of cases with multiple missense variants, as well as a single patient homozygous for an *ATM* variant that was relatively rare in the control population sampled. In 9 patients with HD, three of whom also had BC, more than one missense mutation was observed. In 4 of 9 cases with multiple mutations, one of the alleles observed was the variant D1853N, observed at an allele frequency of 0.12 of controls in this series and 0.18 in another series (30). This variant appears at polymorphic frequencies in healthy populations, and the D1853N allele has also been observed to modify penetrance in germ-line carriers of HNPCC-associated mutations, suggesting a possible interactive effect (39). The P604S/F1463C genotype was documented in 2 cases and 6 controls of identical ethnic origin, suggesting that these alleles may be in linkage disequilibrium in the Ashkenazim. However, in the two cases, but not in the controls, there was a third allele (D1853N or L2307F) present in addition to the P604S/F1463C genotype, consistent with a possible interactive effect of multiple *ATM* missense mutations in a subset of cancer cases. F858L/P1054R, observed in 1 case and 2 controls in this series, was documented as a haplotype in the current series and one prior report (29).

Unlike truncation mutations that lead to formation of unstable protein, missense mutations or short insertion/deletion mutations can potentially lead to formation of a stable but functionally defective protein (40, 41). Multiple missense mutations acting in *cis* may increase the chance of inactivating the atm protein and affect the ability of the protein to serve as a dominant negative to the wild-type allele. Multiple missense mutations found on both chromosomes will increase the chance of inactivating both alleles. Although functional studies of the atm protein in cell lines derived from cases with various combinations of variants are needed to support their pathogenetic role, recent studies have already documented dominant negative effects of *ATM* missense mutations on radiation-induced kinase activity (42, 43).

The variants documented in this report are distributed throughout the open reading frame of the *ATM* genome, but there appears to be a sparing of the COOH-terminus, including the phosphatidylinositol 3′-kinase domains. Mutations in the carboxyl region are associated with the more severe phenotypes of syndromic AT. Missense mutations outside of this region may interfere with *ATM* function, for example, by creation or destruction of glycosylation sites (associated with P604S and S707P, respectively) or by decreased expression of the *ATM* protein by other mechanisms. A lowered expression of the protein product of *ATM* has been correlated with the variant P1054R (31). Recently, an excess of rare *ATM* variants, including the S707P, P1054R, and L1420F alleles seen in lymphoma cases in this series, were documented in a series of unselected BC cases (29). Although their clinical and functional significance remains unclear, multiple rare *ATM* variants may disrupt interactions with other proteins involved in the cellular response to DNA damage, and thus may contribute to the pathogenesis of a subset of human malignancies.

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