ATM Haplotypes and Cellular Response to DNA Damage: Association with Breast Cancer Risk and Clinical Radiosensitivity

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ABSTRACT

The ATM gene, mutated in the cancer-prone and radiation-sensitive syndrome ataxia-telangiectasia (AT), could predispose to breast cancer (BC) development and adverse radiotherapy responses. Sixteen ATM variants were genotyped in 254 BC cases, 70 of whom were adverse radiotherapy responders (RS-BC), and 312 control subjects and the ATM haplotypes were constructed. Constitutive ATM protein, cell survival, and the p53 response after exposure to ionizing radiation were compared in lymphoblastoid cell lines (LCLs) established from the BC cases, AT, and normal individuals.

The tightly linked intronic ATM polymorphisms IVS22–77 T>C and IVS48 + 238 C>G, in the homoyzgote state were associated with increased BC risk [IVS22–77 CC versus TT odds ratio (OR), 1.67; 95% confidence interval (CI), 1.00–2.81], and in the heterozygote state with clinical radiosensitivity [IVS22–77 CT versus TT OR, 0.45; 95% CI, 0.24–0.85]. Homozygote carriers of the G5575A variant were over-represented in RS-BC cases compared with non-RS-BC cases (OR, 6.76; 95% CI, 1.19–38.43). These three single nucleotide polymorphisms were associated with the three major ATM haplotypes present in >80% of the study population. BC LCLs treated with ionizing radiation exhibited an inter-mediate cell survival and p53 response between that of normal and AT LCLs, with the response in the RS-BC LCLs being more compromised than in the non-RS-BC LCLs. Our study suggests a general pattern of increased BC risk associated with carrying any one of the ATM variants studied, with a significant association being observed in individuals carrying variants on both ATM alleles (OR, 1.75; 95% CI, 1.09–2.81) and that ATM variants may impact on radiation sensitivity.

INTRODUCTION

The risk of developing breast cancer (BC) without a background of a family history could be largely explained by the presence of gene mutations or variants of low penetrance more frequently found in the general population than the highly penetrant but rarely mutated genes such as BRCA1 and BRCA2 that confer a high risk of familial BC. The ATM (ataxia telangiectasia mutated) gene, mutated in the recessive cancer-prone disease Ataxia telangiectasia (AT), is one such candidate gene. The ATM gene encodes a protein kinase of 350 kDa, which plays a key role in the detection and the repair of DNA double-strand breaks (1). The ATM protein is mainly nuclear, present as a dimer in human cells. Exposure to ionizing radiation (IR) induces its autophosphorylation on serine 1981 leading to dimer dissociation and the accessibility of the kinase domain (2). The subsequent signaling cascade in which ATM substrates, including p53, Mdm2, Chk2, BRCA1, SMC1, Nbs1, and FanCD2, are phosphorylated results in the activation of cell cycle checkpoints, DNA repair, or the apoptotic process (reviewed in Ref. 3). AT is a rare cancer-prone disease (1 case in 40,000–100,000 live births) with the affected individuals showing a severe clinical phenotype involving cerebellar ataxia, skin and ocular telangiectasias, immunodeficiency, and extreme in vivo and in vitro radiosensitivity (4, 5). AT heterozygotes are estimated to represent ~1% of the general population and while show none of the severe symptoms seen in homozygotes, have a higher mortality rate and an earlier age at death from cancer and ischemic heart disease than noncarriers (6). Several epidemiological studies conducted in AT families have demonstrated a 4-fold increased risk of developing BC among female heterozygotes (Ref. 7 and references therein; Refs. 8–11). On the basis of the heterozygote frequency and increased BC risk seen in these individuals, AT heterozygosity may account for up to 5% of all BC cases in the general population (7). Several studies have attempted to evaluate the frequency of AT heterozygotes in nonfamilial BC cases with different conclusions on the importance of the role of the ATM gene being reached. However, a higher frequency of several ATM sequence variants, including missense alterations and small in-frame deletions or insertions, have been reported in BC cases than in controls (12–16). These results are in agreement with the existence of two groups of AT heterozygotes within the general population, one carrying truncating mutations commonly found in AT patients and the other carrying missense mutations, and with different cancer risks (17).

Because one of the hallmark features of AT is radiosensitivity, it might be expected that BC patients that show clinical radiosensitivity, characterized by severe normal tissue reactions after conventional radiotherapy protocols, might be a group of BC cases with a higher frequency of ATM gene alterations than other BC cases. Indeed, Iannuzzi et al. (18) reported 2 missense or synonymous ATM mutations in 3 of 3 BC patients who showed grade 3–4 s.c. late reactions after radiotherapy, whereas only 3 of 43 patients who did not develop this form of severe toxicity carried an ATM mutation. However, many other such studies have failed to correlate the ATM mutation status with the occurrence of damage in normal tissue (13, 19–23).

To clarify the role of the ATM gene in BC etiology we assessed the frequency of 16 ATM sequence variants in BC cases and controls. Their frequency was also compared between the cases that developed or not an adverse reaction to radiotherapy. Four variants were described previously in the literature, and the others were identified from the screening of the entire ATM coding region in 51 of the 254 cases in the study population. Eleven of the 16 ATM variants were used for haplotype reconstruction, and in a subset of the BC cases for which lymphoblastoid cell lines (LCLs) were available, we explored whether the genotype could be related to the in vitro cellular phenotype.

MATERIALS AND METHODS

Subjects

Local ethical committees approved the study design. The BC cases were recruited over the period of February 1996 to April 2002 from among women...
treated in the Radiotherapy Department at Lyon-Sud Hospital (Pierre-Bénite, France). The average treatment dose was 50 Gy, in dose fractions of 2.5 Gy over a 6-week period, followed by a 10 Gy boost to the tumor bed (24). A total of 254 BC cases over the age of 35 years were enrolled (average age at radiotherapy, 56.8 ± 10.2 years; range, 35–81). Seventy cases (average age at radiotherapy, 58.8 ± 10.8 years; range, 35–81) that displayed adverse reactions to their radiotherapy as classified and graded by the European Organization for Research and Treatment of Cancer (25) in the 2-year period after the start of their radiotherapy formed the radiation sensitive BC (RS-BC) group. This group included those who showed early, early and late, or late normal tissue reactions. None of the remaining 184 BC cases (average age at radiotherapy, 56.1 ± 9.9 years; range, 35–78) developed any adverse reactions to their radiotherapy over a period of 2 years after the start of their treatment (the nonradiation sensitive-non-RS-BC group). The 46 BC cases (27 non-RS-BC and 19 RS-BC) who had received chemotherapy before their radiotherapy were identified by the review of medical records, which were not available for 10 cases (3.8% non-RS-BC and 4.2% RS-BC) of the 254 BC cases. For all of the cases a blood sample was collected after written informed consent had been obtained on the occasion of a routine control visit to the radiotherapy clinic.

Control blood samples (312) were obtained from female blood donors over the age of 35 years living in the catchment area of the hospital, through community-based collections of the Regional Blood Transfusion Service (average age at sample collection, 54.5 ± 6.7 years; range, 35–66). The health status of these controls remained unknown, although it should be noted that any bias from such an individual having cancer would be expected to favor a null result.

**Blood Sample Processing**

Peripheral blood samples were collected using standard venipuncture techniques and were processed within 24 h of collection. The blood samples from the controls were briefly centrifuged (2000 rpm for 10 min), and DNA and RNA were isolated from the buffy coat using Qiagen extraction kits (Qiagen SA., Courtaboeuf, France).

For the BC cases DNA and RNA were isolated from 0.5-ml aliquots of whole blood using Qiagen extraction kits. The lymphocytes were isolated from the remaining of the blood sample using a Ficoll gradient and stored frozen in the presence of 10% DMSO in liquid nitrogen until being used for establishing LCLs.

**LCLs**

As part of this study, LCLs were established by EBV infection for 46 RS and 61 non-RS BC cases. EBV-immortalized LCLs from 8 individuals with a wild-type ATM gene (IARC lines 1104, 1326, 1663, 1665, 2083, 2130, 2145, and 2209), 10 AT patients with a classical clinical phenotype all carrying truncating mutations in the ATM gene (Refs. 26, 27; IARC lines AT3, AT6, AT8, AT11, AT13, AT14, AT173, FRAT3, FRAT12, and GM3189), and 6 obligate AT heterozygote subjects (IARC lines 2362, 2364, 2379, 2383, GM3187, and GM3188) were used for comparative purposes in this study. These lines had been established previously at IARC, or were obtained from Dr. Gilbert Lenoir (Gustave Roussy Institute, Villejuif, France), Dr. Dominique Stoppa-Lyonnet (Curie Institute, Paris, France), or the National Institute of General Medical Science Mutant Cell Repository (Camden, NJ). All of the LCLs were routinely cultured in RPMI 1640 Glutamax-1 medium (Life Technologies, Inc.) supplemented with 10% heat-inactivated fetal calf serum (Intergro b.v, Zaandam, the Netherlands) and 1% penicillin/streptomycin (Biochrom KG, Berlin, Germany) at 37°C under an atmosphere of 5% CO2.

**Analysis of the ATM Gene**

**Mutational Analysis of the ATM Coding Region**

To establish the profile of ATM variants in BC cases the ATM coding region was analyzed from the first 51 sequentially recruited BC cases (14 non-RS-BC and 37 RS-BC cases) for which LCLs were available. This analysis was carried out using the cDNA based protein truncation test (PTT) or restriction endonuclease finger-printing. Total RNA was extracted from the LCLs using the Trizol reagent (Invitrogen). The reaction product was used as a template to amplify the entire ATM coding region using the following primers, ATM A: 5’-atggatctactaatgatgcg-3’ and ATM B: 5’-aaggtgaatgaaaggtat-3’.

The PTT analyses were performed on 37 LCLs by in vitro translation of seven separate, overlapping PCR products (each 1–1.6 kb; primers available on request) covering the ATM coding region, using the TNT T7 Reticulocyte System (Promega, Madison, WI). The PCR product was analyzed by SDS-PAGE electrophoresis and autoradiography (28). The restriction endonuclease finger-printing protocol was carried out on the 37 LCLs analyzed by PTT and 14 additional LCLs as detailed previously (Ref. 29; lists of primers and enzymes are available on request).

PCR products exhibiting an abnormal migration pattern after PTT or restriction endonuclease finger-printing analysis were sequenced using the ABI Prism Big Dye Terminator v2.0 ready reaction cycle sequencing kit (Applied Biosystem) using the exonic described by Sandoval et al. (30).

**Determination of the Frequencies of ATM Sequence Variants in BC Cases and Controls**

The frequency of the 8 ATM sequence changes found in the 51 BC cell lines analyzed by restriction endonuclease finger-printing or PTT was assessed in the remainder of the BC cases and the control population, using a variety of techniques (Table 1). Eight additional ATM variants either previously described in the literature as being BC related or identified in intronic regions flanking exons containing variants identified in the 51 BC cases, were analyzed using a similar approach. A random sample of DNAs was also analyzed by direct sequencing of the corresponding exon as described above, and complete concordance between the different techniques was observed (data not presented).

**Short Oligonucleotide Mass Analysis**

Forty ng of genomic DNA was amplified using up to three sets of primers each specific for a polymorphism and containing the recognition site (CTGGAG) for the type-II restriction endonuclease Bpm1 as described previously (29). The PCR product was digested overnight at 37°C with 5 units of Bpm1 (New England Biolabs, Beverly, MA) or at 30°C with 5 units of its isoenzyme Gsl (Fermentas Inc., Hanover, MD). The 7, 8, or 9-mer oligonucleotides generated were then characterized by electrospray-tandem mass spectrometry based on both the molecular weight and sequence (full technical details available on request).

**RFLP.** The presence of certain polymorphisms was determined using variant specific RFLP using 1 μl of the diluted reverse transcription-PCR product or 200 ng of genomic DNA. When the variant did not create or remove a natural restriction site, primers were designed to set up a PCR-mediated site-directed mutagenesis system, which created an allele-specific restriction endonuclease Bpm1 site as described previously (29). The PCR product was digested overnight at 37°C with 5 units of its isoenzyme Gsl (Fermentas Inc., Hanover, MD). The 7, 8, or 9-mer oligonucleotides generated were then characterized by electrospray-tandem mass spectrometry based on both the molecular weight and sequence (full technical details available on request).

**Techniques used for ATM variant genotyping**

<table>
<thead>
<tr>
<th>Variant</th>
<th>Exon/ Intron</th>
<th>AA* change</th>
<th>Techniques</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVS5016 T&gt;G</td>
<td>10</td>
<td>G707P</td>
<td>RFLP</td>
</tr>
<tr>
<td>T 2119 C</td>
<td>15</td>
<td>F858L</td>
<td>RFLP, SOMA</td>
</tr>
<tr>
<td>T 2572 C</td>
<td>19</td>
<td>L1420F</td>
<td>SOMA, RFLP</td>
</tr>
<tr>
<td>IVS22-77 T&gt;C</td>
<td>22</td>
<td>F1491F</td>
<td>SOMA</td>
</tr>
<tr>
<td>C 3161 G</td>
<td>24</td>
<td>P1054R</td>
<td>SOMA, + sequencing</td>
</tr>
<tr>
<td>C 4148 T</td>
<td>30</td>
<td>S1383L</td>
<td>SOMA, + RFLP</td>
</tr>
<tr>
<td>C 4258 T</td>
<td>31</td>
<td>L1420F</td>
<td>SOMA, + RFLP</td>
</tr>
<tr>
<td>C 4473 T</td>
<td>32</td>
<td>F1491F</td>
<td>SOMA</td>
</tr>
<tr>
<td>C 4578 T</td>
<td>32</td>
<td>P1250P</td>
<td>RFLP</td>
</tr>
<tr>
<td>A 5089 G</td>
<td>36</td>
<td>T1697A</td>
<td>SOMA</td>
</tr>
<tr>
<td>IVS518-15 G&gt;C</td>
<td>38</td>
<td>DHPLC, + sequencing</td>
<td></td>
</tr>
<tr>
<td>IVS518-8 T&gt;C</td>
<td>38</td>
<td>DHPLC, + sequencing</td>
<td></td>
</tr>
<tr>
<td>G 5557 A</td>
<td>39</td>
<td>D1853N</td>
<td>DHPLC, + sequencing</td>
</tr>
<tr>
<td>A 5558 T</td>
<td>39</td>
<td>D1853V</td>
<td>DHPLC, + sequencing</td>
</tr>
<tr>
<td>IVS48+238 C&gt;G</td>
<td>48</td>
<td>RFLP</td>
<td></td>
</tr>
<tr>
<td>T7271G</td>
<td>51</td>
<td>V224AG</td>
<td>SOMA, short oligonucleotide mass analysis.</td>
</tr>
</tbody>
</table>

* AA, amino acid; DHPLC, denaturing high performance liquid chromatography; SOMA, short oligonucleotide mass analysis.

Not detected in the 51 BC cell lines analyzed by restriction endonuclease finger-printing or PTT.
agarose gel. DNA samples known to be carrying the variant allele were included in each analysis, with the genotype of the samples being determined by the banding pattern observed on the gels.

**Denaturing High Performance Liquid Chromatography.** Denaturing high performance liquid chromatography analysis, based on the formation of DNA heteroduplex and homoduplex, was performed on a WAVE DNA Fragment Analysis System (Transgenicom, Omaha, NE). Buffer gradient and temperature conditions were calculated using the WAVEmaker software (version 3.4.4; Transgenicom).

**Estimation of Haplotype Frequencies and Linkage Disequilibrium.** The Hardy-Weinberg equilibrium and linkage disequilibrium were assessed, and the ATM haplotypes were reconstructed, using the 11 SNPs with an allele frequency of >0.5%, using the Genotype Transposer (32) in conjunction with the Arlequin software (33) for all of the BC cases, the RS-BC cases, and the control populations.

**Characterization of BC LCLs**

**Determination of the Basal Level of ATM Protein in Lymphoblastoid Cell Extracts**

Fifty μg of a whole cell protein extract prepared as described previously (34) was separated on SDS-PAGE (6%), transferred onto a polyvinylidene difluoride membrane and immunoblotted with an ATM antibody (1:1000 dilution; AHP392; Serotec, Oxford, United Kingdom) and a Ku80 antibody (1:4000 dilution; AHP317; Serotec). The ATM intensity values were calculated from densitometric analysis of the Western blot autoradiographic films, after correction for protein content using Ku80 as an internal control and normalization against cell lines carrying a wild-type ATM gene. Measurements were done at least twice.

**Cellular Response to DNA Damaging Agents**

**Radiation Survival.** The radiation survival of the LCLs was assessed by comparing their relative growth 72 h after irradiation (35). The cells were seeded at 2 × 10^5 cells/ml the day before irradiation. Cell survival was assessed 3 days after exposure to 0, 1, 2, and 4 Gy with a ^137Cs source, by counting the number of living cells assessed by trypan blue exclusion. The number of viable cells in the nonirradiated cultures was considered as 100%.

For previous exposure to chemotherapy. SNP comparisons were carried out independently of one another. All of the statistical tests were two-sided.

The effect of the genotype on the constitutive ATM expression level and cellular response to DNA damage in the different groups was assessed by the ANOVA. Transformations of the variables were applied to the percentage of surviving cells (arc sine of the square root) and to p53 protein and WAF1/Cip1 (p21) inductions (logarithm) to stabilize the variance when significant heterogeneity was detected by the Bartlett test. The association between the indexes of the cellular response to DNA damage was measured by Pearson’s correlation.

**RESULTS**

**Screening of the ATM Gene.** Only one, established from a RS-BC case, of the 51 BC lines in which the entire ATM coding region was analyzed was found to carry a truncating mutation located in exon 44 (6100 C>T, R2034X). In comparison, 8 different ATM variants, 2 of which have not been described previously in the ATM mutation database² were found in 34 of the 51 cell lines from BC cases examined. To compare the frequency of these ATM gene alterations in the BC cases and control subjects, we genotyped them and 8 additional ATM variants using a variety of techniques. The 2 ATM mutations IVS10–6 >G and T7271G, reported previously to be associated with an increased risk of breast cancer (15, 37–39), were not detected in this series of BC cases. Two ATM variants, C4148T and A5089G, were each detected in 1 of the 254 BC cases and not in the 312 controls, and the T4473C variant was observed in only 2 BC cases and 2 controls (Table 2). Of these 3 variants only the C4148T substitution has been described in the ATM mutation database.

The association between each polymorphism and BC risk or enhanced clinical radiosensitivity is given in Table 2. All of the SNPs are in Hardy-Weinberg equilibrium in both the controls and BC cases. A positive association was found between the highly linked IVS22–77 C and IVS48 + 238 G variant alleles and BC risk, with the homozygote carriers of the rare alleles being more frequently found in the BC cases than in controls (IVS22–77 CC versus TT, OR, 1.67; 95% CI, 1.00–2.81; and IVS48 + 238 GG versus CC OR, 1.66; 95% CI, 1.00–2.76). Heterozygote carriers of these 2 SNPs were found as frequently in the BC cases as in the controls. However, within the BC group there was a significant difference in their distribution with the variant alleles being found more frequently in the non-RS-BC cases than in RS-BC cases (IVS22–77 CC versus TT OR, 0.45; 95% CI, 0.24–0.85 and for IVS48 + 238 CG versus CC OR, 0.50; 95% CI, 0.27–0.94), suggesting a radioprotective effect of these two SNPs when present in the heterozygote state. Homozygote carriers of the G5557A allele were over-represented in the RS-BC cases (5.7%) compared with the non-RS-BC cases (1.1% OR, 6.76; 95% CI, 1.19–38.43). This result suggests that the 5557 genotype may influence the response of BC patients to radiotherapy. No statistically significant differences in the frequency of the other SNPs studied were found between the controls and BC cases, or the non-RS-BC and RS-BC cases. Chemotherapy administered before radiotherapy was significantly associated with the risk of radiosensitivity (OR, 2.2; 95% CI, 1.12–4.30), but did not confound the association with the genetic traits.

**Haplotype Determination.** The 11 SNPs with an allele frequency >0.5% have been used to reconstruct the ATM haplotypes (Table 3). Six major haplotypes (AH1–AH6) were found in the controls and BC cases, with just 3 haplotypes (AH1, AH2, and AH3) representing >80% of the population in both groups. The haplotype AH2, containing the rare alleles of IVS22–77 T>C and IVS48 + 238 C>G,
Table 2. OR* and 95% CI for BC and for clinical radiosensitivity in BC cases by ATM variants

<table>
<thead>
<tr>
<th>Variant</th>
<th>N (n = 312)</th>
<th>BC (n = 254)</th>
<th>OR (95% CI) non-RS-BC (n = 184)</th>
<th>RS-BC (n = 70)</th>
<th>OR (95% CI) NC</th>
</tr>
</thead>
<tbody>
<tr>
<td>T 2119 C</td>
<td>TT 303/248 1.00 178/70 1.00</td>
<td>TC 9/6 0.81 (0.29–2.32) 6/0 NC</td>
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</tr>
<tr>
<td>T 2572 C</td>
<td>TT 297/243 1.00 175/68 1.00</td>
<td>TC 15/11 0.90 (0.40–1.99) 9/2 0.62 (0.13–2.99)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IVS22-77 T&gt;C</td>
<td>TT 136/90 1.00 58/32 1.00</td>
<td>TC 139/123 1.34 (0.93–1.92) 96/27 0.45 (0.24–0.85)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>C 3161 G</td>
<td>CC 288/234 1.00 168/66 1.00</td>
<td>CG 23/20 1.07 (0.57–2.00) 16/4 0.63 (0.20–1.97)</td>
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<tr>
<td>C 4148 T</td>
<td>CC 312/253 1.00 184/69 1.00</td>
<td>CT 0/1 NC 0/1 NC</td>
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<td></td>
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</tr>
<tr>
<td>C 4258 T</td>
<td>CC 307/247 1.00 180/67 1.00</td>
<td>CT 5/7 1.74 (0.55–5.55) 4/3 2.43 (0.52–11.28)</td>
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<tr>
<td>T 4473 C</td>
<td>TT 310/252 1.00 183/69 1.00</td>
<td>TC 2/2 1.23 (0.17–8.80) 1/1 2.13 (0.12–36.61)</td>
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<tr>
<td>C 4578 T</td>
<td>CC 289/232 1.00 169/63 1.00</td>
<td>CT 23/21 1.14 (0.61–2.11) 14/7 0.99 (0.35–2.75)</td>
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</tr>
<tr>
<td>A 5089 G</td>
<td>AA 312/253 1.00 184/69 1.00</td>
<td>AG 0/1 NC 0/1 NC</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>IVS38-15 G&gt;C</td>
<td>GG 307/252 1.00 183/69 1.00</td>
<td>GC 5/2 0.49 (0.09–2.53) 1/1 2.13 (0.12–36.61)</td>
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<tr>
<td>IVS38-8 T&gt;C</td>
<td>TT 295/236 1.00 171/65 1.00</td>
<td>TC 17/18 1.32 (0.68–2.63) 13/5 1.16 (0.39–3.42)</td>
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<tr>
<td>G 5557 A</td>
<td>GG 240/192 1.00 141/51 1.00</td>
<td>GA 65/56 1.08 (0.72–1.61) 41/15 1.02 (0.51–2.04)</td>
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<tr>
<td>A 5558 T</td>
<td>AA 308/247 1.00 179/68 1.00</td>
<td>AT 4/7 2.18 (0.63–7.54) 5/2 0.97 (0.18–5.27)</td>
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<tr>
<td>IVS48+238 C&gt;G</td>
<td>CC 134/89 1.00 58/31 1.00</td>
<td>CG 139/122 1.32 (0.92–1.90) 94/28 0.50 (0.27–0.94)</td>
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<tr>
<td>Other</td>
<td>Variable</td>
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</table>

* OR, odds ratio; CI, confidence interval; BC, breast cancer; RS, radiation sensitive; NC, not calculated.

Table 3. Reconstructed ATM haplotypes

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Sequence*</th>
<th>Control</th>
<th>All BC**</th>
<th>OR (95% CI) non-RS-BC</th>
<th>RS-BC</th>
<th>OR (95% CI)</th>
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</thead>
<tbody>
<tr>
<td>AH1</td>
<td>TTTCCCTGAG</td>
<td>293/200</td>
<td>1.00</td>
<td>137/63</td>
<td>1.00</td>
<td>0.62 (0.39–0.99)</td>
</tr>
<tr>
<td>AH2</td>
<td>TTTCCCTGAG</td>
<td>180/175</td>
<td>1.42 (1.08–1.88)</td>
<td>136/39</td>
<td>1.00</td>
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<tr>
<td>AH3</td>
<td>TTTCCCTGAG</td>
<td>62/50</td>
<td>1.18 (0.78–1.79)</td>
<td>33/17</td>
<td>1.12 (0.58–2.16)</td>
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</tr>
<tr>
<td>AH4</td>
<td>TTTCCCTGAG</td>
<td>21/20</td>
<td>1.40 (0.74–2.64)</td>
<td>14/6</td>
<td>0.93 (0.34–2.54)</td>
<td></td>
</tr>
<tr>
<td>AH5</td>
<td>TTTCCCTGAG</td>
<td>16/17</td>
<td>1.56 (0.77–3.15)</td>
<td>13/4</td>
<td>0.67 (0.21–2.13)</td>
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<tr>
<td>AH6</td>
<td>TTTCCCTGAG</td>
<td>15/10</td>
<td>0.97 (0.43–2.22)</td>
<td>9/1</td>
<td>0.24 (0.03–1.95)</td>
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</tr>
<tr>
<td>AH7</td>
<td>TTTCCCTGAG</td>
<td>9/6</td>
<td>0.97 (0.34–2.79)</td>
<td>6/0</td>
<td>NC</td>
<td></td>
</tr>
<tr>
<td>AH8</td>
<td>TTTCCCTGAG</td>
<td>8/8</td>
<td>1.47 (0.54–3.97)</td>
<td>7/1</td>
<td>0.31 (0.04–2.58)</td>
<td></td>
</tr>
<tr>
<td>AH9</td>
<td>TTTCCCTGAG</td>
<td>5/2</td>
<td>0.59 (0.11–3.05)</td>
<td>1/1</td>
<td>2.18 (0.13–35.35)</td>
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</tr>
<tr>
<td>AH10</td>
<td>TTTCCCTGAG</td>
<td>5/5</td>
<td>1.47 (0.42–5.13)</td>
<td>3/2</td>
<td>1.45 (0.24–8.90)</td>
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<tr>
<td>AH11</td>
<td>TTTCCCTGAG</td>
<td>4/3</td>
<td>1.10 (0.24–4.96)</td>
<td>2/1</td>
<td>1.09 (0.10–12.22)</td>
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<tr>
<td>AH12</td>
<td>TTTCCCTGAG</td>
<td>3/6</td>
<td>2.93 (0.72–11.86)</td>
<td>5/1</td>
<td>0.43 (0.05–3.80)</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>Variable</td>
<td>3/6</td>
<td>2.93 (0.72–11.86)</td>
<td>5/1</td>
<td>0.43 (0.05–3.80)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>624/508</td>
<td>368/140</td>
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* The nucleotide positions and bp changes defining the haplotypes are T2119C, T2572C, IVS22-77 T>C, C3161G, C4258T, C4578T, IVS38-15 G>C, IVS38-8 T>C, G5557A, A5558T, and IVS48+238 C>G. Base changes from the wild-type sequence are indicated in bold and underlined.

** BC, breast cancer; OR, odds ratio; CI, confidence interval; RS, radiation sensitive; NC, not calculated.

was found more frequently in the BC cases compared with the controls (BC versus controls OR, 1.42; 95% CI, 1.08–1.88) and in particular in the non-RS-BC cases (RS-BC versus non-RS-BC OR, 0.62; 95% CI, 0.39–0.99) confirming the associations described above. Taking into the account the genotype of each individual we also noted that excess risk of BC was greatest in homozygote carriers of the AH2 haplotype (OR, 2.03; 95% CI, 1.09–3.81). A pattern of increased BC risk was observed for carriers of a haplotype containing at least one of the ATM variants studied, although this did not reach statistical significance (OR, 1.44; 95% CI, 0.95–2.2). Individuals who carried ATM variants on both alleles instead showed a significant increased BC risk (OR, 1.75; 95% CI, 1.09–2.81).
RS-BC cases. Containing the T2119C variant allele, which was not found in the similarly represented in the controls and BC cases except AH7, RS-BC cases than in non-RS-BC cases. The other haplotypes were this 5557A rare allele, were not observed more frequently in the homozygote state, the AH3 and AH5 haplotypes, which contain this 5557A rare allele, were not observed more frequently in the RS-BC cases than in non-RS-BC cases. The other haplotypes were similarly represented in the controls and BC cases except AH7, containing the T2119C variant allele, which was not found in the RS-BC cases.

Characterization of the Cellular Response to IR. To examine the possible biological effects of these ATM variants, we characterized the cellular response to DNA damage in LCLs established from non-RS-BC and RS-BC cases, compared with LCLs from control and AT subjects carrying ATM truncating mutations. In a limited number of cell lines we explored whether a correlation between genotype and cellular phenotype existed. A relative growth measurement was used as a surrogate for cell survival in the LCLs (35). Using this approach the cell survival of the 47 BC cell lines studied was intermediate between that of the 7 controls and the 8 AT LCLs at all of the doses examined (Fig. 1). The LCLs from the 26 non-RS-BC cases are indistinguishable from those established from the 21 RS-BC cases after exposure to 1 Gy (RS versus non-RS, P = 0.238). However, after exposure to 2 or 4 Gy, the LCLs from the RS-BC cases appeared significantly more sensitive to cell killing than those from the non-RS-BC cases (RS versus non-RS; P = 0.00002 at 2 Gy; P = 0.033 at 4 Gy). Under these experimental conditions the survival of the 5 AT heterozygote cell lines was similar to that seen for the LCLs from the non-RS-BC cases, and was distinguishable from the control and AT LCLs after 2 and 4 Gy. After exposure to 4 Gy the cell survival of the cell lines from the RS-BC cases was lower than that seen for the LCLs from the AT heterozygote lines (non-RS versus AT het, P = 0.097; RS versus AT het, P = 0.003).

The induction of p53 protein and WAF1/Cip1(p21) mRNA levels after exposure to IR is a well-established measure of the functionality of the ATM signaling pathway with AT cell lines showing a compromised response (40). Analysis of the p53 protein and WAF1/Cip1(p21) mRNA induction 3 h after 2 Gy irradiation allowed only the response between the 7 control LCLs and the 8 AT cell lines to be distinguished as has been reported previously (41). However, the cellular responses 2 and 4 h after exposure to 5 Gy of IR showed significant (P < 0.05) cell type-specific differences. Adjusting the results for time after exposure, the degree of p53 protein and WAF1/Cip1(p21) mRNA induction was significantly lower in the AT and the 30 RS-BC LCLs compared with the control and the 13 non-RS-BC LCLs, with the levels in the 3 AT heterozygote LCLs being intermediate between that of the control and the AT LCLs, and indistinguishable from the RS-BC LCLs (Fig. 2). A very good correlation was observed between the induction levels of p53 protein and WAF1/Cip1(p21) mRNA in the cell lines examined when the results were adjusted for time after exposure (P < 0.0001).

The constitutive level of ATM protein in the BC LCLs does not seem to be the underlying cause of the variability of the cellular response to DNA damage observed in these cell lines. The level of ATM protein did not correlate either with the in vitro cell survival (r² = 0.00; P = 0.474) or with the induction level of the p53 protein (r² = 0.00; P = 0.981) or WAF1/Cip1(p21) mRNA (r² = 0.00; P = 0.855) after exposure to IR. The constitutive ATM protein level

Fig. 1. Cell survival of LCLs. Radiation survival of LCLs established from control, AT, AT heterozygote (AT het) and RS- or non-RS-BC cases was measured by relative growth at 72 h after 0, 1, 2, and 4 Gy irradiation. Each point represents the mean of the total number (n) of each cell type analyzed. bars, ±SD. The number of viable cells in the nonirradiated culture 72 h after irradiation was considered as 100% cell survival.

Fig. 2. Cellular response to DNA damage in LCLs. Fold induction in p53 protein (A) and WAF1/Cip1 (p21) mRNA (B) expression after exposure to 2 or 5 Gy ionizing radiation in LCLs established from control, AT, AT heterozygote (AT het), and RS- or non-RS-BC cases. Each value represents the mean of the total number (n) of each cell type analyzed.
was also not different between the cell lines from the 59 non-RS-BC and 44 RS-BC cases ($P = 0.578$).

**Genotype/Phenotype Correlations.** The relationship between genotype and cellular phenotype has been investigated in a limited number of cell lines. The cell lines from the BC cases carrying different SNPs ($P = 0.807$) or carriers of the rare ATM variants C4148T, T4473C, and A5089G showed no differences in the constitutive ATM protein level. The BC cell line carrying the heterozygote truncating mutation had a 3-fold lower level of ATM protein than the normal LCLs. No significant differences were observed in the constitutive ATM protein level ($P = 0.226$), the cell survival ($P = 0.329$), or the p53 protein induction ($P = 0.158$) after IR exposure in the cell lines carrying the variant G5557A in the wild-type, heterozygote, or homozygote state.

As discussed above the comparison of the cell survival between the two groups of BC cases showed that at doses above 2 Gy the LCLs established from the RS-BC cases had a significantly lower survival compared with the LCLs from the non-RS-BC cases. However, when this comparison was made between the LCLs established from the 6 RS-BC and 5 non-RS-BC cases carrying the IVS22–77C/IVS48 + 238G variant allele (AH2 haplotype) in the heterozygote state, there was no difference in the dose-adjusted relative cell growth measured 72 h after irradiation ($P = 0.105$), suggesting a radioprotective effect of this SNP in the LCLs from the RS-BC cases in agreement with the association study results. The 5 LCLs from the clinically RS-BC cases carrying this haplotype also exhibited a lower p53 induction than the 6 LCLs from the RS-BC cases carrying the wild-type AH1 haplotype ($P = 0.0165$).

**DISCUSSION**

In this study we have assessed the association between ATM haplotypes and breast cancer risk and enhanced clinical radiosensitivity, and investigated the possible correlation between the cellular response to IR exposure of LCLs established from BC cases and their ATM genotype. On the basis of the analysis of the entire ATM cDNA we found only one carrier of a truncating mutation in the 51 BC cases studied, in agreement with the low prevalence of this type of mutations in sporadic BC cases and the reported frequency, of ~1%, of AT heterozygotes in the general population (4, 21, 42, 43). Only one study has reported previously a higher frequency of ATM germ-line truncating mutations in a BC population. Broeks et al. (37) found that 6 of 7 ATM mutations, identified in 82 Dutch breast cancer cases characterized by frequent bilateral occurrence, early age of onset, and long-term survival, were truncating mutations. Three of these were the IVS10–6T>G mutation, which has been associated with an increased risk for breast cancer both in the general population and in high-risk breast cancer families, and which has been traced back to a single ancient mutational event at least 50,000 years ago (44).

Several ATM missense variants including 3 rare variants, 2 of which have not been reported previously in the ATM mutation database, were detected in this present study. Only analysis of the functional impact of these variants on ATM activity will determine whether they should be considered as mutations or rare polymorphisms. We have not detected any of the specific SNPs or their combinations associated with BC risk as reported previously (15, 37–39, 45). Two recent studies were also negative for the C3161G or the T2119C variant alleles (46, 47). However, the two highly linked polymorphisms, IVS22–77 T>C and IVS48 + 238 C>G, were associated with an increased risk of breast cancer when present in the homozygote state. IVS48 + 238 C>G has only been studied previously in relation to BC survival and was not found to be a determinant (48). Our study had a very low statistical power for most of the associations examined for the individual ATM SNPs. Nevertheless, we did observe a general pattern of increased BC risk associated with carrying a haplotype containing any one of the ATM variants studied, which whereas it did not reach statistical significance, deserves testing in larger studies. Moreover, a significant association with BC risk was noted in those individuals carrying variants on both ATM alleles confirming a role for the ATM gene as a BC risk factor.

In our study, cases and controls were recruited from different sources, although from the same residential area. Information on socioeconomic factors were not available to control for possible selection bias. However, the fact that the genotype distributions of cases and controls were in Hardy-Weinberg equilibrium suggests comparability of the two groups. Another limitation of the study is the lack of information on established risk factors for BC. These include factors related to reproductive history, use of exogenous hormones, and anthropometric characteristics linked to habitual diet (49), and it is planned to overcome these limitations in the continuation of this study.

The association between a specific genotype and the increased risk of developing a cancer raises the question of the functional relevance of the variant allele. To date, few studies have analyzed the functional consequences of the presence of variants on ATM function. The T7271G and IVS10–6 T>G transversions have been associated with an increased risk of breast cancer. In vivo analysis of p53-serine 15 and BRCA1 phosphorylation in LCLs established from heterozygote carriers has revealed that these missense mutations act in a dominant-negative manner with the wild-type ATM kinase unable to function normally in the presence of the mutant protein. The variant S2592C, first detected in a breast cancer patient, has also been defined as a pathogenic missense mutation based on alterations in ATM function (50). The mechanistic basis for the present findings of an increased BC risk associated with the IVS22–77 T>C/IVS48 + 238 C>G SNPs in the homozygote state remains unclear. The intronic variant IVS48 + 238 C>G appears to generate a very weak additional donor splice site (data not presented); however, no additional transcripts have been detected experimentally in the cell lines carrying this variant. The levels of the ATM mRNA transcript in LCLs carrying these variants have been compared using a semiquantitative real-time PCR approach. The preliminary results suggest that a statistically lower level of transcript is present in the variant carriers [wild-type ATM (5 lines) versus heterozygote (5 lines), $P = 0.0005$; wild-type ATM (5 lines) versus homozygote (4 lines), $P = 0.0009$]. The high level of linkage disequilibrium across the ATM gene could also mean that the “causal” variant remains to be determined. Indeed, Bonnen et al. (51) have identified 6 other intronic SNPs that are in linkage disequilibrium with IVS22–77 T>C, as is the IVS10–294 G>A variant detected in this present study (data not presented). Intronic variants in other genes have also been associated with an increased risk of BC, for instance, the p21-intron 2 polymorphism and the CYP19-intron 4[TTTA]$_{19}$ repeat allele (52–54). However, to date no functional studies have been reported linking an altered protein function or cellular phenotype with the presence of these two polymorphisms.

Of the 16 SNPs studied, 2 showed differences in their allele frequencies between the RS and non-RS-BC cases. The presence of the IVS22–77 T>C/IVS48 + 238 C>G SNPs in the homozygote state was more frequently found in the non-RS-BC cases than in the RS-BC cases suggesting an association of these SNPs with a radio-

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9 Personal communication.
protective effect against fibrosis in the normal tissue in the radiation field. These associations were independent of having received chemotheraphy, which as was also reported by Fiets et al. (55), enhances the risk of developing an adverse reaction to radiotherapy. This association is supported by the results from the cell survival characteristics of the LCLs established from this group of individuals. In general the LCLs established from the BC cases showed an intermediate cell survival after exposure to IR between that of the normal and the AT cell lines but similar to that seen in the AT heterozygote cell lines. A lower response in the LCLs established from RS-BC cases compared with the LCLs from non-RS-BC cases was also noted. This suggests that genetic alterations influencing these end points may be present in the BC LCLs, with some being specific to the cell lines established from the RS-BC cases and associated with this in vitro phenotype. In the LCLs carrying the IVS22–77C/IVS48 + 238G variant allele (AH2 haplotype) associated with the wild-type allele (AH1 haplotype), no difference in the cell survival after exposure to IR was observed between those lines established from the 6 RS-BC and the 5 non-RS-BC cases, providing evidence for a radioprotective effect of this haplotype at the lymphocyte level only in the RS-BC LCLs and experimental support of the SNP association studies. The few LCLs examined, however, means that the results should be interpreted with caution.

The G5557A variant in the homozygote state was, in contrast, associated with enhanced clinical radiosensitivity in breast cancer patients, although no in vitro cellular phenotype has been associated with the presence of this variant in the LCLs studied. A preliminary report on the frequency of AT heterozygotes among prostate cancer patients also found a higher proportion of carriers of an exon 39 polymorphism (G5557A) in radiosensitive prostate cancer cases (46%) than in cancer cases who had no severe late effects after radiation therapy (18%) suggesting that this ATM variant might be considered as one risk factor predisposing to adverse normal tissue reactions after radiotherapy. This polymorphism has also been shown to influence the penetrance of hereditary nonpolyposis colorectal cancer in carriers of germ-line MLH1 and MSH2 mutations (57). To date, no genetic factors that might specifically influence the temporal occurrence of these adverse reactions have been identified, nor is there evidence to suggest that the genetic determinants of early or late reactions are different. Unfortunately, the few individuals within the RS-BC group presenting early, early and late, or late effects does not allow us to assess whether the presence of these variant alleles can impact differentially on the temporal occurrence of these adverse tissue reactions. Approximately 10% of cancer patients show some degree of adverse tissue reactions in response to radiotherapy with the underlying causes of these interindividual variations remaining to be established (58). Genetically determined intrinsic differences related to the presence of mutations or polymorphisms in genes normally responsible for the detection and the processing of DNA damage produced by IR might greatly influence the interindividual variation seen in the responses to radiation therapy (59, 60). For example, a polymorphism in the hHR21 gene (61), and the association of variant alleles in exon 6 (codon 194) and exon 10 (codon 399) in the XRCCI gene have been found recently to be associated with an enhanced clinical radiosensitivity (62). Determining such genetic markers could be helpful in tailoring the treatment dose for each patient, as to date no screening techniques are available that predict normal tissue reactions to conventional radiotherapy protocols.

In our study, we found three major ATM haplotypes representing >80% of the chromosomes studied. This result shows considerable overlap with the ATM haplotypes described previously by Bonnen et al. (51) and Thorstenson et al. (63) in which an ancestral chimpanzee ATM sequence was used to determine the root of phylogeny (AH1; Fig. 3). In another recent study (64), the ATM haplotypes were reconstructed based on the genotyping of 6 SNPs in 159 individuals from 83 families with deleterious BRCA1 mutations with similar profiles being found to those described previously. Li et al. (64) examining the ATM haplotypes in AT patients and non-AT patients also found three haplotypes accounting for 93% of all of the chromosomes studied. It should be noted that the population we have analyzed is more homogenous than that of Bonnen et al. (51) or Thorstenson et al. (63), which could explain in part the difference in the number and the frequency of haplotypes observed between these studies (Fig. 3).

The associations that we report need to be confirmed in larger studies in different populations, ideally also accounting for classical risk factors for BC. If confirmed, the genotyping of the IVS22–77 T>C or IVS48 + 238 C>G and G5557A polymorphisms could be
helpful in the identification of women at increased risk of breast cancer and those who might develop adverse tissue reactions after radiotherapy. The LCLs established from the BC cases carrying the same ATM haplotypes but derived from RS or non-RS-BC cases will be a valuable tool for the identification of gene modifiers capable of influencing the cellular response to IR.

ACKNOWLEDGMENTS

We thank Dr. Marina Artuso and Henriette Brésil for their participation in the early stages of this project, Anne-Laure Bertoli and Marie-Thérèse Coulet for their help in breast cancer patient recruitment, and Michelene Absi from the Blood Transfusion Service for help in the recruitment of control subjects. We thank Drs. Olga Sinilnikova, Ruggero Montesano, and Yossi Shiloh for helpful discussions and advice.

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ATM Haplotypes and Cellular Response to DNA Damage: Association with Breast Cancer Risk and Clinical Radiosensitivity

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Cancer Res 2003;63:8717-8725.

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