The effects of Atm haploinsufficiency on mutation rate in the mouse germline and somatic tissue

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Abstract
Using single-molecule PCR, the frequency of spontaneous and radiation-induced mutation at an expanded simple tandem repeat (ESTR) locus was studied in DNA samples extracted from sperm and bone marrow of Atm knock-out (Atm⁻/⁻) heterozygous male mice. The frequency of spontaneous mutation in sperm and bone marrow in Atm⁺/⁻ males did not significantly differ from that in wild-type BALB/c mice. Acute exposure to 1 Gy of γ-rays did not affect ESTR mutation frequency in bone marrow and resulted in similar increases in sperm samples taken from Atm⁺/⁻ and BALB/c males. Taken together, these results suggest that the Atm haploinsufficiency analysed in our study does not affect spontaneous and radiation-induced ESTR mutation frequency in mice.
Introduction

The ATM kinase plays a crucial role in the recognition of spontaneous and radiation-induced double strand (DSB) DNA breaks (1). Mutations at the ATM gene result in the human recessive genetic disorder ataxia-telangiectasia, characterised by genomic instability and extreme sensitivity to ionising radiation and DSB-induced mutagens (1). The results of some recent studies have also shown that heterozygosity for ATM mutations is associated with the increased risk of breast cancer in human populations (2). Given the high frequency of ATM<sup>-/-</sup> heterozygous carriers in humans (3), thorough analysis of their predisposition to cancer and sensitivity to ionising radiation is therefore clearly warranted. To further characterise the effects of ATM deficiency, a number of Atm knock-out (KO) mice have been generated (4-6). In line with the human data, the analysis of Atm KO heterozygous mice has revealed elevated cancer risk (7) and predisposition to radiation-induced cataracts (8), as well as increased radiosensitivity (9). However, the data on the effects of Atm haploinsufficiency on genome stability still remain controversial. Although our studies have shown the elevated level of non-repaired radiation-induced DSBs (10) and chromatid aberrations (11) in the mouse Atm<sup>+/−</sup> cells, the analysis of autosomal mutation induction failed to detect significant differences between the irradiated Atm<sup>−/−</sup> and wild-type mice (12). The results of recent publication suggest that Atm deficiency does not affect the frequency of spontaneous and radiation-induced inversions in heterozygous mice (13). The lack of measurable effects of Atm haploinsufficiency on the frequency of spontaneous homologous recombination in mice was also reported (14).

In our previous studies, we have analysed the germline effects of several DNA repair deficiencies on spontaneous and radiation-induced mutation rates at expanded simple tandem repeat (ESTR) DNA loci (15-19). Using the same approach, here we have studied the effects of Atm haploinsufficiency on spontaneous and radiation-induced mutation in the germline and somatic tissue.

Materials and methods

Materials

The Expand High Fidelity PCR System for Single-Molecule PCR was from Roche, (Mannheim, Germany). A 100 bp DNA Step Ladder was obtained from Promega (Madison, WI, USA). Other reagents and enzymes were obtained from Amersham Biosciences (Little Chalfont, UK), New England Biolabs (Hitchin, UK), Sigma-Aldrich Company Ltd (Poole, UK) and Genetic Research Instruments (Braintree, UK).

Mice

129S6/SvEvTac Atm<sup>tm1Awb</sup> mice originally created by Barlow et al. (4) served as the donor strain for the Atm knockout allele used in this study. BALB/cByJ Atm<sup>tm1Awb</sup> congenic mice were generated by thirteen generations of conventional backcrosses followed by five intercross generations. Wild-type BALB/c male mice were purchased from Harlan, UK. Given that Atm<sup>−/−</sup> male mice are sterile (4), all experiments on the genetic effects of Atm deficiency on were therefore carried out on Atm<sup>+/−</sup> heterozygotes. Seven weeks old Atm<sup>+/−</sup> and BALB/c male mice were acutely exposed to whole body irradiation with 1 Gy of 137Cs γ-rays and sacrificed 9 weeks after exposure ensuring that the sperm collected was derived from irradiated A<sub>y</sub> spermatogonal stem cells (20). Control animals were age-matched to the irradiated males. All animal procedures were carried out under the Home Office project license No. PPL 80/1564, and CSU IACUC protocols 03-132ABC and 05-284A.

DNA Isolation and ESTR Typing

DNA samples were prepared in a laminar flow hood as previously described (21, 22). Sperm cells were taken from caudal epididymus. Approximately 500 ng of each DNA sample was
digested with 20 U MseI (New England Biolabs) for at least 2 hours at 37°C; MseI cleaves outside the Ms6-hm locus array and the PCR primer sites.

The frequency of ESTR mutation was evaluated using a single-molecule PCR (SM-PCR) approach (21, 22). DNA was amplified on an MJ DNA engine PTC 220 in 10 µl reactions using 0.6 µM flanking primers Hm1.1f (5’ – AGA GTT TCT AGT TGC TGCA – 3’) and Hm1.R (5’ – GAG AGT CAG TTC TAA GGC AT – 3’), 1 U enzyme mix (Expanded High Fidelity PCR system, Roche), 1 M Betaine and 200 µM dNTPs. After denaturing at 96°C for 3 min, PCRs were cycled at 96°C for 20 sec, 58°C for 30 sec, and 68°C for 3 min for 30 cycles, ending with 10-min incubation at 68°C. To increase the robustness of the estimates of individual ESTR mutation frequencies, on average 120 amplifiable molecules were analysed for each tissue for each male mouse.

PCR products were resolved on a 40 cm long agarose gel and detected by Southern blot hybridisation as previously described (23). The frequencies of ESTR mutation, 95% confidence intervals and standard errors were estimated using modified approach proposed by Chakraborty (24). DNA fragment sizes were estimated by the method of Southern (25), with a 100 bp DNA Step Ladder included on all gels.

Results and discussion
Using SM-PCR (21, 22), the frequency of ESTR mutation at the Ms6-hm locus was evaluated in DNA samples extracted from sperm and the bone marrow tissue of non-exposed and irradiated male mice. This approach involves diluting bulk genomic DNA and amplifying multiple samples of DNA, each containing approximately one amplifiable ESTR molecule. The mean progenitor allele sizes in Atm+/− and BALB/c were similar (2.3–2.5 KB). As used in our previous studies, only bands showing a shift of at least 1 mm relative to the progenitor allele were scored as mutants (Figure 1A).

Table 1 presents a summary of ESTR mutation data. In both tissues the frequency of ESTR mutation in non-irradiated Atm+/− and BALB/c males did not significantly differ. These data are in line with the results of previous studies showing the lack of measurable effects of Atm haploinsufficiency on spontaneous homologous recombination in mice (14). Exposure to ionising radiation resulted in a similar 2.1-2.5 fold increase in ESTR mutation frequency in sperm of Atm+/− and BALB/c males (Figure 1B). The frequency of radiation-induced mutation in Atm+/− and BALB/c males did not significantly differ (Table 1). In this respect, the effects of Atm haploinsufficiency on ESTR mutation are close to those in p53, Msh2 and Xpc deficient mice, where spontaneous and radiation-induced mutation rates in the germline of heterozygotes and wild-type males are indistinguishable (16, 18, 19). Our data are also consistent with the results showing that Atm haploinsufficiency does not affect spontaneous and radiation-induced somatic mutation rates at the mouse Aprt protein-coding gene (12).

In contrast to the sperm data, exposure to ionising radiation did not affect the frequency of ESTR mutation in the bone marrow tissue of Atm+/− and BALB/c males (Figure 1C). These data are in line with the results of our previous study showing the lack of significant increases in ESTR mutation frequencies in the somatic tissues of irradiated male mice (21). The absence of measurable changes in ESTR mutation frequency in bone marrow is consistent with the results showing that radiation-induced ESTR mutations in the mouse germline can only occur in mitotically-proficient spermatogonia (23, 26). Given the very low percentage of stem cells in bone marrow (9.1 per 10⁵ cells in BALB/c mice, ref 27), it would appear that DNA samples extracted from this tissue may be enriched by the genomes of non-dividing supporting cells. If the same mechanisms underlie spontaneous and radiation-induced ESTR mutation in the mouse germline and somatic tissues, then it seems unlikely that the SM-PCR technique can detect increases in mutation frequency in DNA samples taken from the somatic tissues of irradiated adult mice. In addition, the sampling of bone
marrow tissue 9 weeks after irradiation could partially explain the lack of measurable changes in the exposed animals, as at this time point the majority of mature blood cells that are derived from the directly irradiated stem cells should be already in circulation or even gone. To effectively assess ESTR mutation induction in bone marrow tissue, either the flow-sorted fraction of directly exposed stem cells or nucleated blood cells would need to be profiled at an earlier time point.

The incidence of ESTR mutations involving gain or loss was defined for 291 ESTR mutations found in sperm and bone marrow of non-exposed and irradiated BALB/c and Atm+/− males (Table 1). In two groups (irradiated sperm and non-exposed bone marrow) the incidence of ESTR mutations involving gain or loss of repeat units did not significantly differ between BALB/c and Atm+/− mice. In contrast, the frequency of losses in the Atm+/− DNA samples extracted from sperm of non-irradiated and bone marrow of irradiated Atm+/− mice significantly exceeded that in the wild-type strain. However, despite the lack of significant difference in the former groups, which was most probably related to a quite low number of mutations, the frequency of mutations involving loss of repeats exceeded that for gains across all tissues of Atm+/− males. Overall, the total incidence of losses in Atm+/− mice was significantly elevated (67.2% and 42.8% of losses for Atm+/− and BALB/c males, respectively; \( \chi^2 = 16.27; \) df=1, \( P=0.0001 \)). Given that according to our previous results the frequency of gains and the germline of irradiated and non-exposed DNA-repair deficient mice does not significantly differ from that in the wild-type strains (15-19), the Atm+/− data are quite unexpected and remain unexplained.

We next determined the spectra of ESTR mutations. This analysis was restricted by the resolution of agarose gel electrophoresis and the smallest mutational change detected in DNA samples taken from either Atm+/− or BALB/c mice corresponded to the gain or loss of 2 repeats (Figure 1D). Within each genotype the mutation spectra for the exposed and non-irradiated males did not significantly differ (data not shown). The combined distributions of length changes at ESTR loci were indistinguishable between the two strains (Figure 1D). We therefore conclude that neither the Atm haploinsufficiency, nor exposure to ionising radiation affect the length of ESTR mutation changes.

In conclusion, here we have shown that the effects of Atm haploinsufficiency on spontaneous and radiation-induced ESTR mutation rate in heterozygous male are likely to be negligible. These results, however, do not imply that the stability of ESTR loci in Atm+/− homozygotes is not compromised. Given the important role of the ATM protein in double-strand break repair, it is possible to speculate that spontaneous and induced ESTR mutation rates in these animals may be elevated. As ataxia-telangiectasia belongs to the class of genomic instability syndromes and homozygous carries display an abnormally high frequency of chromosome aberrations (1), this may imply that Atm deficiency could also affect ESTR mutation in the Atm+/− KO mice. Such a notion is further supported by the results of our previous study showing highly elevated ESTR mutation rate in the germline of homozygous scid mice (15), which are deficient in the recognition and repair of double-strand breaks by the non-homologous end joining pathway. However, given that in contrast to the scid mice, Atm+/− mice are sterile (4), the effects of Atm deficiency on spontaneous and radiation-induced ESTR mutation can only be analysed in their somatic tissues.

It should also be stressed that the KO mice used in this study may not be the most appropriate experimental model for the effects of ATM deficiency in heterozygous human carriers. We and others have shown that the BALB/c mouse strain carries a hypomorphic allele of Prkdc, the gene encoding the catalytic subunit of DNA-dependent protein kinase (28, 29). This hypomorphic allele diminishes DNA double strand break repair capacity. In line with these data, our previous results show that spontaneous and radiation-induced ESTR mutation rate in the germline of BALB/c significantly exceeds that in other inbred strains.
(30), which might obscure the effects of Atm haploinsufficiency on this genetic background. Also, in contrast to the majority of known mutations at the human ATM gene, the Atm KO used in our study, which was generated by targeted disruption of a 178 bp exon, produces a highly unstable and undetectable protein which does not interact with the product of the wild-type allele (4). In this respect, the Atm KO (Atm-ΔSRI) harbouring a mutation that is common in people with ataxia telangiectasia represents better model as the ΔSRI mutant expresses relatively stable protein with abolished ATM kinase activity and has a dominant-negative effect and mice carrying this mutation have a higher risk of cancer (6, 7). Future studies should analyse the effects of this mutation on genome stability.

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References


**Figure legend**

**Fig. 1.** ESTR mutation frequencies and spectra in irradiated and control male mice. (A) Mutation detection at the *Ms6-hm* locus by SM-PCR in DNA sample containing two progenitor alleles. Mutants are indicated with arrowhead. The frequency of ESTR mutation in DNA samples extracted from sperm (B) and the bone marrow tissue (C). The 95% confidence intervals (CI) of are shown. For each genotype, the probability of difference between control and irradiated males (Student’s test) are given. (D) Spectrum of ESTR mutations in sperm and bone marrow of BALB/c and *Atm<sup>+/−</sup>* male mice (Kolmogorov-Smirnov two-sample test, *P*=0.9855). The progenitor allele was assumed to be the allele closest in size to the mutant allele.
Figure 1
Table 1  
Summary of mutation data

<table>
<thead>
<tr>
<th>Tissue, strain, exposure</th>
<th>No males</th>
<th>No mutations</th>
<th>No progenitors</th>
<th>Frequency ± s.e.(^a)</th>
<th>Ratio(^b)</th>
<th>(t)</th>
<th>(P)(^c)</th>
<th>Type of mutants (%)</th>
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<tr>
<td>Sperm, 0 Gy</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BALB/c</td>
<td>9</td>
<td>60</td>
<td>1350</td>
<td>0.0444 ± 0.0059</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>37 (61.7) 23 (38.3)</td>
</tr>
<tr>
<td>(Atm^{+/−})</td>
<td>2</td>
<td>16</td>
<td>269</td>
<td>0.0594 ± 0.0155</td>
<td>1.34</td>
<td>0.90</td>
<td>0.3682</td>
<td>5 (31.2) 11 (68.8)</td>
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<tr>
<td>(χ^2), df=1(^d)</td>
<td></td>
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<td>4.60 (P=0.0320)</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>BALB/c</td>
<td>3</td>
<td>37</td>
<td>435</td>
<td>0.0851 ± 0.0149</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>19 (51.4) 18 (48.6)</td>
</tr>
<tr>
<td>(Atm^{+/−})</td>
<td>3</td>
<td>45</td>
<td>360</td>
<td>0.1251 ± 0.0202</td>
<td>1.47</td>
<td>1.60</td>
<td>0.1100</td>
<td>16 (35.6) 29 (64.4)</td>
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<tr>
<td>(χ^2), df=1(^d)</td>
<td></td>
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<td>2.04 (P=0.1532)</td>
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<tr>
<td>BALB/c</td>
<td>9</td>
<td>60</td>
<td>1450</td>
<td>0.0414 ± 0.0055</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>31 (51.7) 29 (48.3)</td>
</tr>
<tr>
<td>(Atm^{+/−})</td>
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<td>20</td>
<td>489</td>
<td>0.0409 ± 0.0095</td>
<td>0.99</td>
<td>0.04</td>
<td>0.9681</td>
<td>9 (45.0) 11 (55.0)</td>
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<tr>
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<td></td>
<td>0.26 (P=0.6101)</td>
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<tr>
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<tr>
<td>BALB/c</td>
<td>3</td>
<td>30</td>
<td>480</td>
<td>0.0626 ± 0.0120</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>20 (66.7) 10 (33.3)</td>
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<tr>
<td>(Atm^{+/−})</td>
<td>4</td>
<td>23</td>
<td>482</td>
<td>0.0477 ± 0.0103</td>
<td>0.76</td>
<td>0.94</td>
<td>0.3474</td>
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<td>13.17 (P=0.0003)</td>
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</table>

\(^a\) ± standard error.  
\(^b\) Ratio to BALB/c males.  
\(^c\) Student’s test and probability for difference from BALB/c males.  
\(^d\) Chi-square test for homogeneity of the type of mutants between BALB/c \(Atm^{+/−}\) males.