The effects of *Atm* haploinsufficiency on mutation rate in the mouse germ line and somatic tissue

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Using single-molecule polymerase chain reaction, 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* knockout (*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.

Materials and methods

Materials

The Expand High Fidelity PCR System for single-molecule polymerase chain reaction (SM-PCR) was obtained 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*^+/−^ mice originally created by Barlow et al. (4) served as the donor strain for the *Atm* KO allele used in this study. BALB/cByJ *Atm*^−/−^ congenic mice were generated by 13 generations of conventional backcrosses followed by five intercross generations. Wild-type BALB/c male mice were purchased from Harlan, Bicester, UK. Given that *Atm*^−/−^ male mice are sterile (4), all experiments on the genetic effects of *Atm* deficiency were therefore carried out on *Atm*^+/−^ heterozygotes. Seven-week-old *Atm*^+/−^ 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, spermatogonial stem cells (20). Control animals were age matched to the irradiated males. All animal procedures were carried out under the Home Office project licence 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 epididymis. Approximately 500 ng of each DNA sample was digested with 20 U Msel (New England Biolabs) for at least 2 h at 37°C. Msel cleaves outside the Ms6-hm locus array and the PCR primer sites.

The frequency of ESTR mutation was evaluated using an 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 Hm1.1R (5′-AGAGTTTCTAGTTGCTGTGA-3′) and Hm1.1L (5′-GAGATGTCAGTTCTAAGCCAT-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 hybridization 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.

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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<sup>+/−</sup> 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 I presents a summary of ESTR mutation data. In both tissues, the frequency of ESTR mutation in non-irradiated Atm<sup>+/−</sup> 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 ionizing radiation resulted in a similar 2.1- to 2.5-fold increase in ESTR mutation frequency in sperm of Atm<sup>+/−</sup> and BALB/c males (Figure 1B). The frequency of radiation-induced mutation in Atm<sup>+/−</sup> and BALB/c males did not significantly differ (Table I). In this respect, the effects of Atm haploinsufficiency on ESTR mutation are close to those in p53<sup>−/−</sup>, Msh2<sup>−/−</sup>, and Xpc<sup>−/−</sup>-deficient mice, where spontaneous and radiation-induced mutation rates in the germ line 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 ionizing radiation did not affect the frequency of ESTR mutation in the bone marrow tissue of Atm<sup>+/−</sup> 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 germ line can only occur in mitotically proficient spermatogonia (23,26). Given the very low percentage of stem cells in bone marrow [9.1/10<sup>5</sup> cells in BALB/c mice (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 germ line 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...
Atm haploinsufficiency

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 I). In two groups (irradiated sperm and non-irradiated 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 losses in the germ line 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 two 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 ionizing 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 DSB 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 germ line of homozygous scid mice (15), which are deficient in the recognition and repair of DSBs 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 DSB repair capacity. In line with these data, our previous results show that spontaneous and radiation-induced ESTR mutation rate in the germ line 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

### Table I. Summary of mutation data

<table>
<thead>
<tr>
<th>Tissue, strain, exposure</th>
<th>No. of males</th>
<th>No. of mutations</th>
<th>No. of progenitors</th>
<th>Frequency ± SE&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Ratio&lt;sup&gt;b&lt;/sup&gt;</th>
<th>r&lt;sup&gt;c&lt;/sup&gt;</th>
<th>P&lt;sup&gt;d&lt;/sup&gt;</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 Atm+/−</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)</td>
</tr>
<tr>
<td>Balb/c 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)</td>
</tr>
<tr>
<td>Sperm, 1 Gy</td>
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<td></td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>BALB/c Atm+/−</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)</td>
</tr>
<tr>
<td>Balb/c 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)</td>
</tr>
<tr>
<td>Bone marrow, 0 Gy</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>BALB/c Atm+/−</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)</td>
</tr>
<tr>
<td>Balb/c Atm+/−</td>
<td>3</td>
<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)</td>
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<tr>
<td>Bone marrow, 1 Gy</td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>BALB/c Atm+/−</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)</td>
</tr>
<tr>
<td>Balb/c 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>
<td>4 (17.4)</td>
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<sup>a</sup>± standard error.<br><sup>b</sup>Ratio to BALB/c males.<br><sup>c</sup>Student’s test and probability for difference from BALB/c males.<br><sup>d</sup>Chi-square test for homogeneity of the type of mutants between BALB/c Atm+/− males.
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