

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.

Introduction

The ATM kinase plays a crucial role in the recognition of spontaneous and radiation-induced DNA double-strand breaks (DSBs) (1). Mutations at the *ATM* gene result in the human recessive genetic disorder ataxia-telangiectasia, characterized by genomic instability and extreme sensitivity to ionizing 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*^{+/-} heterozygous carriers in humans (3), thorough analysis of their predisposition to cancer and sensitivity to ionizing radiation is therefore clearly warranted. To further characterize the effects of *ATM* deficiency, a number of *Atm* knockout (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*^{+/-} cells, the analysis of autosomal mutation induction failed to detect significant differences between the irradiated *Atm*^{+/-} 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 germ line 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 germ line and somatic tissue.

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 (Baintree, UK).

Mice

129S6/SvEvTac *Atm*^{tm1Awb} 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*^{tm1Awb} 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_s 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 MseI (New England Biolabs) for at least 2 h at 37°C; MseI cleaves outside the *Msi6-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 μ l reactions using 0.6 μ M flanking primers Hm1.1f (5'-AGAGTTTCTAGTTGCTGTGA-3') and Hm1.R (5'-GAGAGTCAGTTCTAAGGCAT-3'), 1 U enzyme mix (Expanded High Fidelity PCR System, Roche), 1 M betaine and 200 μ M 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*^{+/-} 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*^{+/-} 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*^{+/-} and BALB/c males (Figure 1B). The frequency of radiation-induced mutation in *Atm*^{+/-} 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*-, *Msh2*- and *Xpc*-deficient mice, where spontaneous and radiation-induced mutation rates in the germ line of hetero-

zygotes 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*^{+/-} 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 [$9.1/10^5$ 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

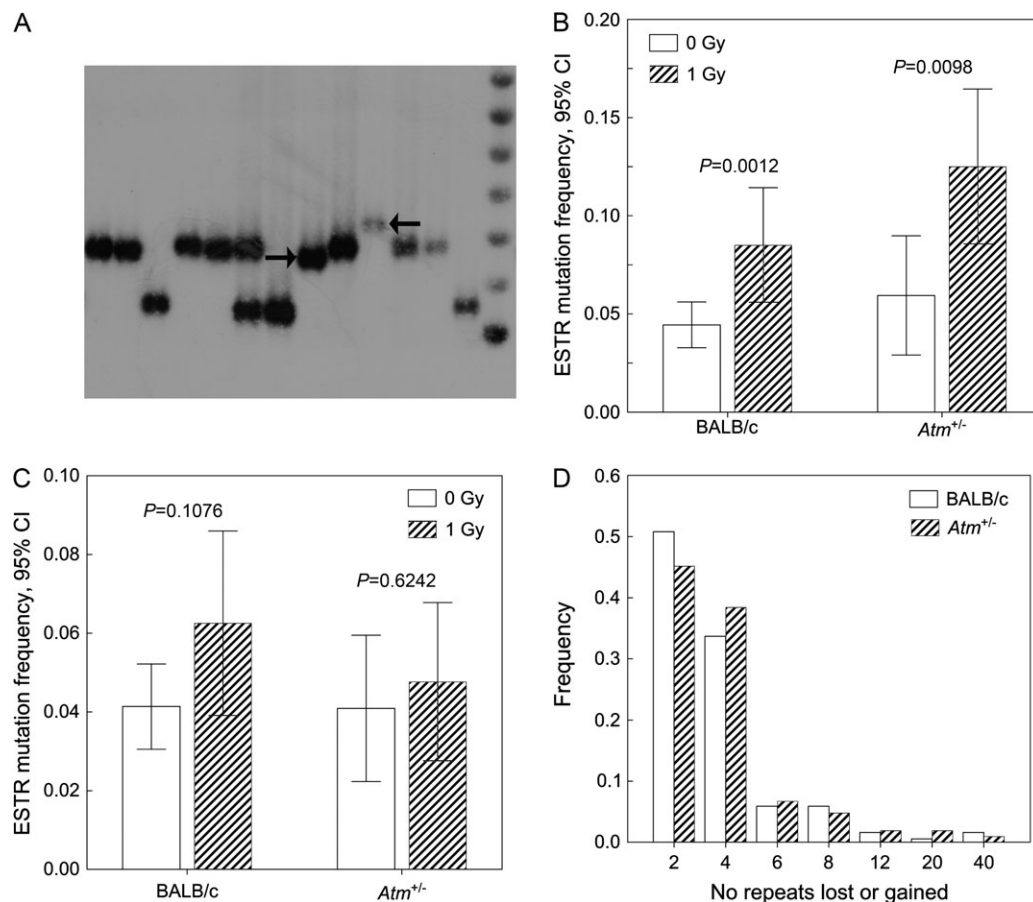


Fig. 1. ESTR mutation frequencies and spectra in irradiated and control male mice. (A) Mutation detection at the *Ms6-hm* locus by SM-PCR in DNA sample containing two progenitor alleles. Mutants are indicated with arrowhead. The frequency of ESTR mutation in DNA samples extracted from sperm (B) and the bone marrow tissue (C). The 95% confidence intervals (CI) 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*^{+/-} 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.

Table I. Summary of mutation data

Tissue, strain, exposure	No. of males	No. of mutations	No. of progenitors	Frequency $\pm$ SE ^a	Ratio ^b	<i>t</i> ^c	<i>P</i> ^c	Type of mutants (%)	
								Gains	Losses
Sperm, 0 Gy									
BALB/c	9	60	1350	0.0444 $\pm$ 0.0059	–	–	–	37 (61.7)	23 (38.3)
<i>Atm</i> ^{+/-}	2	16	269	0.0594 $\pm$ 0.0155	1.34	0.90	0.3682	5 (31.2)	11 (68.8)
χ^2 , df = 1 ^d								4.60	<i>P</i> = 0.0320
Sperm, 1 Gy									
BALB/c	3	37	435	0.0851 $\pm$ 0.0149	–	–	–	19 (51.4)	18 (48.6)
<i>Atm</i> ^{+/-}	3	45	360	0.1251 $\pm$ 0.0202	1.47	1.60	0.1100	16 (35.6)	29 (64.4)
χ^2 , df = 1 ^d								2.04	<i>P</i> = 0.1532
Bone marrow, 0 Gy									
BALB/c	9	60	1450	0.0414 $\pm$ 0.0055	–	–	–	31 (51.7)	29 (48.3)
<i>Atm</i> ^{+/-}	3	20	489	0.0409 $\pm$ 0.0095	0.99	0.04	0.9681	9 (45.0)	11 (55.0)
χ^2 , df = 1 ^d								0.26	<i>P</i> = 0.6101
Bone marrow, 1 Gy									
BALB/c	3	30	480	0.0626 $\pm$ 0.0120	–	–	–	20 (66.7)	10 (33.3)
<i>Atm</i> ^{+/-}	4	23	482	0.0477 $\pm$ 0.0103	0.76	0.94	0.3474	4 (17.4)	19 (82.6)
χ^2 , df = 1 ^d								13.17	<i>P</i> = 0.0003

^a $\pm$ standard error.^bRatio to BALB/c males.^cStudent's test and probability for difference from BALB/c males.^dChi-square test for homogeneity of the type of mutants between BALB/c *Atm*^{+/-} males.

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 I). 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 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

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*- Δ SRI) harbouring a mutation that is common in people with ataxia-telangiectasia represents better model as the Δ SRI 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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