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The effects of *in utero* irradiation on mutation induction and transgenerational instability in mice

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ABSTRACT

Epidemiological evidence suggests that the deleterious effects of prenatal irradiation can manifest during childhood, resulting in an increased risk of leukaemia and solid cancers after birth. However, the mechanisms underlying the long-term effects of foetal irradiation remain poorly understood. This study was designed to analyse the impact of *in utero* irradiation on mutation rates at expanded simple tandem repeat (ESTR) DNA loci in directly exposed mice and their first-generation (F_1) offspring. ESTR mutation frequencies in the germline and somatic tissues of male and female mice irradiated at 12 days of gestation remained highly elevated during adulthood, which was mainly attributed to a significant increase in the frequency of singleton mutations. The prevalence of singleton mutations in directly exposed mice suggests that foetal irradiation results in genomic instability manifested both *in utero* and during adulthood. The frequency of ESTR mutation in the F_1 offspring of prenatally irradiated male mice was equally elevated across all tissues, which suggests that foetal exposure results in transgenerational genomic instability. In contrast, maternal *in utero* exposure did not affect the F_1 stability. Our data imply that the passive erasure of epigenetic marks in the maternal genome can diminish the transgenerational effects of foetal irradiation and therefore provide important clues to the still unknown mechanisms of radiation-induced genomic instability. The results of this study offer a plausible explanation for the effects of *in utero* irradiation on the risk of leukaemia and solid cancers after birth.

1. Introduction

The developing embryo is especially sensitive to ionising radiation, exposure to which results in foetal mortality and increases the risk of leukaemia and solid cancers after birth [1,2]. However, to date the mechanisms underlying the health risks associated with prenatal irradiation remain poorly understood. Given that the majority of embryonic cells are actively proliferating, exposure during the early stages of development could lead to a substantial accumulation of radiation-induced mutations across all tissues, thus contributing to the risk of cancer after birth. It is therefore clear that the analysis of mutation induction *in utero* can provide important insights into the long-term effects of foetal exposure to mutagens, including ionising radiation. It should however be stressed that at present the genetic effects of prenatal irradiation remain poorly characterised. To date, the analysis of both gene mutations and chromosome aberrations in the somatic tissues of adult mice irradiated *in utero* has resulted in a highly controversial set of data. A number of studies have shown that the frequency of chromosome aberrations and gene mutations in haemopoietic cells remained elevated during adulthood following foetal irradiation [3–5], while in others no measurable increases in translocation frequencies were found in the lymphocytes of *in utero* exposed adults [6,7]. Although relatively few studies have analysed the impact of *in utero* exposure on the developing germline, preliminary evidence suggests a low efficiency for radiation-induced mutations in foetal gonads [8,9]. One major reason for the apparently conflicting results describing the effects of foetal irradiation is due to the lack of a sensitive *in vivo* technique that efficiently detects spontaneous and radiation-induced mutations in both the germline and somatic tissues. Further analysis of *in utero* mutation induction, using an appropriate detection system, therefore offers an opportunity to gain novel insights into the effects of prenatal exposure to mutagenic agents. One such system, that we have used extensively, utilises mouse expanded simple tandem repeat (ESTR) loci as a biomarker of mutation induction in germline and somatic cells following adult exposure to many mutagenic agents, including ionising radiation [10–13]. These studies have shown that ESTR loci provide a sensitive experimental system for monitoring germline mutation in mice, permitting evaluation of mutation induction at low doses of exposure and in very small population samples.

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In addition to the studies of mutation induction in the germline and somatic tissues of directly exposed organisms, considerable progress has been made in the analysis of the transgenerational effects of parental irradiation, which are manifested in the offspring of treated parents (reviewed in Refs. [14–17]). It was established that mutation rates in the germline and somatic tissues of non-treated offspring of adult male exposed to ionising radiation or chemical mutagens remain highly elevated [17–21]. These data suggest that transgenerational genomic instability may be a contributory factor in the elevated cancer risk and enhanced tumour progression observed in the offspring of exposed fathers. They also indicate that a transgenerational genomic destabilisation of the genome can be attributed to an as yet unknown radiation-induced signal in the paternal genome which is inherited through sperm in an epigenetic fashion. In previous studies we have evaluated the transgenerational effects of irradiation of different stages of mouse spermatogenesis and found that mutation rates remained equally elevated in the offspring conceived from 1 to 8 weeks after paternal exposure [18–20]. These results imply that a radiation-induced instability signal is retained during spermatogenesis, even after several rounds of DNA replication. The persistence of such a signal in the adult germline raises questions regarding the effects of *in utero* exposure on transgenerational instability. It should however be stressed that the massive epigenetic reprogramming occurring in the developing germline [22] could potentially erase all epigenetic marks of foetal radiation exposure, thus preventing the manifestation of genomic instability in subsequent generations.

Using a combination of approaches to assess ESTR mutation rate, here we have studied the effects of *in utero* irradiation on mutation induction and transgenerational instability in mice.

2. Materials and methods

2.1. Animals

BALB/c mice were obtained from Harlan (Bicester, UK) and housed at the Division of Biomedical Services, University of Leicester. Eight-week-old pregnant females (12 days of gestation) and 7-week-old adult males were given whole-body acute irradiation of 1 Gy of X-rays delivered at 0.5 Gy min^{-1} (250 kV constant potential, HLV 1.5 mm Cu, Pantak industrial X-ray machine, Connecticut, USA). Eight-week-old *in utero* irradiated and sham-treated male and female mice were mated to non-irradiated BALB/c partners (Fig. 1A). Tissue samples were taken from all irradiated mice and from the 8-week-old male offspring of *in utero* irradiated parents. All animal procedures were carried out under the Home Office project license No. PPL 80/1564.

2.2. ESTR mutation detection in pedigrees

Genomic DNA was prepared from tails by SDS lysis, proteinase K digestion, phenol/chloroform extraction and ethanol precipitation. All parents and offspring were profiled using two mouse-specific hypervariable single-locus ESTR probes Ms6-hm and Hm-2 as described previously [10]. DNA samples were resolved on a 40-cm long agarose gel and detected by Southern blot hybridization. Autoradiographs were scored by two independent observers. As in a number of previous studies [11–14,23], only bands showing a shift of at least 1 mm relative to the progenitor allele were scored as mutants. DNA fragment sizes were estimated by the method of Southern [24], with a 1 kb DNA Ladder (Invitrogen, Paisley, UK) included on all gels.

2.3. ESTR mutation detection by single-molecule PCR

To minimise the risk of contamination, DNA samples from sperm, bone marrow (BM) and brain were prepared in a laminar flow hood as previously described [11,19]. Briefly, the sperm pellet was suspended in 1× SSC and somatic cells lysed by addition of SDS to 0.15%. The lysate was centrifuged and the resulting sperm pellet re-suspended in 1 ml 0.2× SSC, 1% SDS, 1 M 2-mercaptoethanol and digested with 200 μg ml^{-1} proteinase K (Sigma) for 1 h at 37 °C. DNA was recovered after phenol/chloroform extraction. DNA from BM and brain was similarly prepared by SDS lysis, proteinase K digestion, phenol/chloroform extraction and ethanol precipitation.

Approximately 5 μg of each DNA sample was digested with 20 U MseI for 2 h at 37 °C; MseI cleaves outside the ESTR array and distal to the PCR primer sites used for PCR amplification and was used to render genomic DNA fully soluble prior to dilution. Each digested DNA sample was diluted to approximately 10 ng ml^{-1} in 5 mM Tris–HCl (pH 7.5) in the presence of 5 μg ml^{-1} carrier salmon sperm DNA (Sigma, Dorset, UK) prior to mutation analysis.

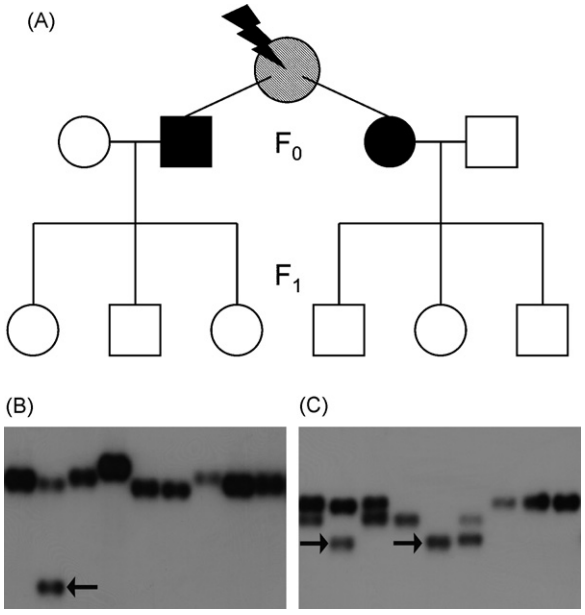

Fig. 1. Experimental design and mutation detection at the *Ms6-hm* locus by SM-PCR. (A) Design of *in utero* study. Irradiated pregnant female is in grey; *in utero* exposed parents (F_0) are in black; their non-exposed offspring (F_1) and control parents with no history of irradiation are in white. (B) Representative image of a singleton ESTR mutation. (C) Representative image of a mosaic ESTR mutation. Mutants are indicated with arrowheads.

The frequency of ESTR mutation was evaluated using SM-PCR as previously described [11,19]. The *Ms6-hm* ESTR locus was amplified in 10 μl reactions using 0.4 μM flanking primers HM1.1F (5′-AGA GTT TCT AGT TGC TGT GA-3′) and HM1.1R (5′-GAG AGT CAG TTC TAA GGC AT-3′). Amplification was performed using 0.035 U μl^{-1} of the Expand High Fidelity PCR System (Roche, Mannheim, Germany), 1 M Betaine and 200 μM each dNTP. Amplification was carried out at 96 °C (20 s), 58 °C (30 s), 68 °C (8 min) for 30 cycles on a PTC-225 DNA Engine Tetrad (BioRad, Hemel Hempstead, UK). To increase the robustness of the estimates of individual ESTR mutation frequencies, on average 140 amplifiable molecules were analysed for each tissue for each mouse.

PCR products were resolved on a 40-cm long agarose gel and detected by Southern blot hybridization as previously described [10]. Following Southern blot hybridization, autoradiographs were scored by two independent observers. The frequencies of ESTR mutation and standard errors were estimated using a modified approach proposed by Chakraborty and coworkers [25].

3. Results

3.1. Experimental design

As in a number of our previous studies [18–21], we have used BALB/c inbred mice, to evaluate the effects of *in utero* irradiation on ESTR mutation induction and transgenerational instability. BALB/c pregnant mice (Theiler stage 20, 12 days of gestation) were exposed to 1 Gy of acute X-rays. At this stage of mouse development, primordial male and female germ cells are already in the genital ridge area and undergo active mitotic proliferation before entering meiotic or mitotic arrest at about 13.5 days post coitum [26]. The majority of somatic cells also undergo active mitotic proliferation in 12-day mouse embryos [27].

Using a pedigree-based approach, we first evaluated ESTR mutation rates in the germline of *in utero* irradiated males and females. The *in utero* irradiated mice (generation F_0) were mated to non-exposed partners and ESTR germline mutations were scored in their first-generation (F_1) offspring (Fig. 1A). To compare the effects of *in utero* paternal irradiation with adult exposure, 7-week-old male mice were given 1 Gy of acute X-rays and mated to control females 8 weeks after irradiation, thus ensuring that their offspring were