Germline minisatellite mutations in workers occupationally exposed to radiation at the Sellafield nuclear facility

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Received 16 April 2014, revised 21 July 2014
Accepted for publication 27 August 2014
Published 8 December 2014

Abstract

Germline minisatellite mutation rates were investigated in male workers occupationally exposed to radiation at the Sellafield nuclear facility. DNA samples from 160 families with 255 offspring were analysed for mutations at eight hypervariable minisatellite loci (\textit{B6.7, CEB1, CEB15, CEB25, CEB36, MS1, MS31, MS32}) by Southern hybridisation. No significant difference was observed between the paternal mutation rate of 5.0\% (37 mutations in 736 alleles) for control fathers with a mean preconceptional testicular dose of 9 mSv and that of 5.8\% (66 in 1137 alleles) for exposed fathers with a mean preconceptional testicular dose of 194 mSv. Subgrouping the exposed fathers into two dose groups with means of 111 mSv and 274 mSv revealed paternal mutation rates of 6.0\% (32 mutations in 536 alleles) and 5.7\% (34 mutations in 601 alleles), respectively, neither of which was significantly different in comparisons with the rate for the control fathers. Maternal mutation rates of 1.6\% (12 mutations in 742 alleles) for the partners of control fathers and 1.7\% (19 mutations in 1133 alleles) for partners of exposed fathers

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were not significantly different. This study provides evidence that paternal preconceptional occupational radiation exposure does not increase the germline minisatellite mutation rate and therefore refutes suggestions that such exposure could result in a destabilisation of the germline that can be passed on to future generations.

Keywords: minisatellite loci, germline mutation, occupational radiation exposure

(Some figures may appear in colour only in the online journal)

1. Introduction

The health consequences of exposure to ionising radiation gained prominence in the aftermath of the dropping of the atomic bombs on Hiroshima and Nagasaki in 1945. Animal studies had indicated that radiation exposure could cause genetic effects and it was recognised that man was unlikely to be exempt (Muller 1941). The first concern, therefore, was to develop population based family studies to evaluate the incidence of disorders with a genetic component in the offspring of the atomic bomb survivors (Neel and Schull 1953, Schull et al 1981, Schull 2003). These studies continue but, as yet, no evidence of an increase in germline mutagenesis has been observed (Neel et al 1990, Neel 1998). In contrast to the lack of genetic effects, it soon became apparent that the incidence of a number of malignant diseases, most notably leukaemia, was increased in the exposed population (Schull 1998) and, therefore, the focus of subsequent studies of radiation-exposed populations shifted to the evaluation of somatic effects in those directly exposed. Thus, when the UK nuclear energy programme was developed, the National Registry for Radiation Workers was established (Goodwin 1975) to enable epidemiological studies of potential radiation-induced diseases in the workers.

Interest in the heritable effects of exposure to radiation regained prominence when Gardner et al (1990) reported that the raised incidence of leukaemia and non-Hodgkin’s lymphoma (NHL) observed among children living near the Sellafield nuclear reprocessing plant was associated with the recorded occupational dose of paternal preconceptional external whole body radiation received while working at Sellafield. It was further suggested that radiation-induced mutations occurring in the paternal germ cells had caused a predisposition to leukaemia or NHL in the next generation. This suggestion was controversial for two main reasons. Firstly, it implied a level of risk inconsistent with the accepted risks for genetic disease in humans and secondly, there was little evidence that germline mutation was a significant cause of childhood leukaemia (Abrahamson 1990, Evans 1990, Narod 1990, Baverstock 1991, Neel 1991).

In the absence of any clear evidence for radiation-induced hereditary effects in humans (Boice 2003, Schull 2003, Wyrobek et al 2007, Signorello et al 2012, Little et al 2013, Salomaa et al 2013), risks of radiation-induced genetic disease are derived by applying mouse data on radiation-induced mutation rates to human data on spontaneous frequencies of genetic diseases (United Nations Scientific Committee on the Effects of Atomic Radiation (UNSCEAR) 2001, Biological Effects of Ionizing Radiation (BEIR) VII 2006, International Commission on Radiological Protection (ICRP) 2007). Spontaneous mutation rates at loci associated with human genetic diseases are very low and the findings of Gardner et al (1990) added impetus to the use of molecular technology to identify other novel genetic markers of radiation-induced germline mutation which might allow a better quantification of radiation human genetic risk.

One such approach is the analysis of minisatellite mutations at hypervariable loci. Minisatellites are tandem repeat loci typically 0.5–30 kbp long with repeat units in the
range 6–100 bp. They occur at high frequency in the human genome, are preferentially located near the ends of chromosomes, and frequently show variability in repeat copy number and therefore allele length (Jeffreys et al 1995). Some of these repeat DNA sequences exhibit high frequencies of spontaneous germline mutations to new allele lengths and it has been suggested that screening for length changes provides the opportunity to detect induced germline mutations using relatively small population samples (Dubrova et al 1996, Dubrova 2003a).

Initial studies of minisatellite mutations in populations contaminated by the Chernobyl accident in Belarus (Dubrova et al 1996, 1997) and Ukraine (Dubrova et al 2002a) reported statistically significant increases in paternal, but not maternal, mutation rates in the offspring of those exposed, which correlated with estimated environmental contamination with radio-nuclides. In a similar study, a significantly increased rate of paternal minisatellite mutation, which extended to a second generation, was observed in the germlines of irradiated parents living around the Semipalatinsk nuclear test site in Kazakhstan (Dubrova et al 2002b). At around the same time, work on the induction of germline mutations in tandem repeat loci in irradiated mice confirmed that the increase in mutations observed in the offspring was maintained in the subsequent generation (Barber et al 2002). It was suggested that this increase in mutations was indicative of a more general radiation-induced genomic instability (Niwa and Kominami 2001) and that this could provide an explanation for the observed excess of leukaemia and NHL associated with offspring of fathers working at the Sellafield facility (Barber et al 2002, Dubrova 2003b).

This work was included in a review by the Committee on Medical Aspects of Radiation in the Environment (COMARE) (2002) which examined the evidence concerning the incidence of cancer in the offspring of parents occupationally exposed to radiation prior to the conception of their children. It was noted that studies in mice had shown that radiation damage in male germ cells can be extended to DNA sequences that have not been subject to direct radiation damage and also to sequences in descendant unirradiated cells, thus amplifying the effect of the initial radiation damage. This could, in principle, result in enhanced sensitivity of the offspring to a further carcinogen. Whilst recognising that it was not known whether the same mechanisms would work in man, it was speculated that an interaction between such changes and a specific as yet unknown factor was not impossible, and could explain the high incidence of leukaemia and NHL in children living close to Sellafield.

Against this background of continuing uncertainty and speculation regarding the impact of preconceptional paternal occupational exposure on germline mutation and adverse health outcomes in the next generation, a study was initiated to establish the minisatellite mutation rate in the offspring of workers occupationally exposed to radiation at the Sellafield nuclear facility. The results of this study are presented here.

2. Materials and methods

2.1. Study group

The retired male radiation workers included in this study of minisatellite mutations were part of a wider investigation which also examined in vivo chromosome aberrations in relation to recorded occupational dose (to be reported separately) and in vitro chromosome radiosensitivity (previously reported (Cadwell et al 2008)). The rationale for this was to reduce intrusion caused by multiple visits and to use a single blood sample for all studies. Prior to commencement, a presentation was made to workforce representatives at the Sellafield facility which outlined the reasons for the study and how it would be undertaken.
Having gained their support, ethical approval was obtained from North Cumbria Local Research Ethics Committee in May 2003 and the study started in January 2004. Initially, the men contacted had been part of a previous chromosome aberration study of retired workers (Tawn et al 2004) and had expressed a willingness to be contacted again. However, in 2005 it was realised that this approach was not providing the necessary range of doses and permission was sought from the Local Research Ethics Committee to identify more workers from the Sellafield dosimetry records, and also to include current workers. Approval was received in May 2005. Enrolment in the study and collection of blood samples continued until September 2008. Letters were sent to 825 radiation workers and included an information sheet describing the studies and a reply/consent form. Separate permission was sought for participation in the different studies and also for the banking of DNA and lymphocytes for future studies. Potential participants were chosen on the basis of their recorded occupational radiation exposure histories at the time and because they were living locally. Two study groups were initially identified based on total cumulative occupational doses from external sources of radiation as measured by film badge readings (Kite and Britcher 1996), a control group with doses <50 mSv and an exposed group with doses >100 mSv. A small number of men with known intakes of plutonium as measured by urine analysis (Riddell et al 2000) were also contacted. It was notable that a number of men were happy to participate themselves but did not want their families to be involved and therefore there was a greater uptake for the chromosome work than for the minisatellite study. Of the 825 men contacted only 175 agreed to take part in the minisatellite study, an uptake of 21%. The study genetic nurse then visited the participants in their homes, taking the partner’s consent form. The study was further explained and blood samples taken from the radiation worker and his partner. In addition, at this visit, permission was sought to approach the offspring. Letters, information sheets and reply/consent forms were then sent to the offspring, and those consenting were visited by the study genetic nurse who took blood samples. If grandchildren were available, written permission to participate was obtained from their parents and the children themselves, and blood samples taken.

Of the 175 men who initially agreed to participate with their families, four families were unsuitable because family members were not available and one excluded because the radiation worker had received radiotherapy prior to conception of his offspring. In addition, 10 families were the subject of technical failure, mainly due to the low concentration of the DNA sample. In some families with more than one offspring, not all agreed or were available or suitable and samples from some individual offspring were technically unsuccessful. Thus the final study group comprised 160 families with 255 offspring and 23 grandchildren. Following identification of the final study group, individual dosimetry records were re-examined to determine total preconceptional gonadal doses, i.e. cumulative doses up to 38.5 weeks before the birth of each child. This resulted in some men who had originally been identified as having external doses >100 mSv being reclassified as controls since their exposure had mainly occurred following the conception of their children. Moreover, the small number of men originally identified as having significant lifetime doses from intakes of plutonium were all found to have had little or no such exposure prior to conception of any offspring and, therefore, there was nothing to distinguish them from the rest of the larger group. They were treated the same way as the other workers and their offspring allocated to control or exposed groups based on total preconceptional gonadal dose. In all cases the radiation doses received by the men in this study were accumulated over their working lives. Some fathers with more than one offspring appeared in both groups because they had received additional exposure between conceptions. The control group comprised 103 offspring and 10 grandchildren and the exposed group 152 offspring and 13 grandchildren. Each of the families was assigned a laboratory number and
each sample given an additional suffix -01 for father, -02 for mother and -03, -04, -05 and so on for offspring and -03G1, -03G2, -04G1 and so on for grandchildren. Information on dose category was not known during the laboratory analysis.

2.2. Confirmation of parentage

For confirmation of maternity, paternity and sample identity, the Applied Biosystems (Warrington, UK) AmpFlSTR COfiler PCR amplification kit, which amplifies six tetranucleotide short tandem repeat loci (D3S1358, D16S539, TH01, TPOX, CSF1PO, D7S820) plus a segment of the sex-specific amelogenin locus was utilised. Semi-automated analysis of PCR products was carried out on an ABI 310 genetic analyser platform, the data files generated were then analysed using Genotyper 2.X software (Applied Biosystems). For both procedures, a mismatch between parent and offspring at two or more loci was considered to be a non-paternity or non-maternity. One set of monozygotic twins was identified and treated in the data set as one offspring.

2.3. Preparation of minisatellite probes

Minisatellite mutations were analysed using eight single locus probes by Southern blotting. Hypervariable loci were selected according to their high background mutation frequency. Probes used were B6.7 (20q13), CEB1 (D2S90), CEB15 (D1S172), CEB25 (D10S180), CEB36 (D10S473), MS1 (D1S7), MS31 (D7S21) and MS32 (D1S8). B6.7 and CEB probes were made by PCR amplification of alleles <5 kb, according to methods provided by Professor Yuri Dubrova, University of Leicester, UK. PCR products were purified and ligated into the cloning vector pGEM-T easy (Promega, Southampton, UK) and transformed into XL-10 Gold Ultracompetent cells (Stratagene Europe, Amsterdam, The Netherlands). Plasmid DNA was extracted from 150 ml bacterial cultures using Hi-Speed Maxi Prep kits (Qiagen, West Sussex, UK) followed by gel extraction using a QiaQuick gel extraction kit (Qiagen). MS probes were a gift from Professor Alec Jeffreys, University of Leicester, UK.

2.4. Minisatellite mutation analysis

Five micrograms of genomic DNA was digested with restriction enzyme Alu I (New England Biolabs) and electrophoresed on a 30 cm 0.8% agarose gel in 1 x Tris-Borate-EDTA (ethylenediaminetetraacetic acid) (TBE) buffer (Sigma-Aldrich Company Ltd, Dorset, UK) containing 0.5 µg ml⁻¹ ethidium bromide (Sigma-Aldrich) overnight at 110 V to separate. The DNA was then denatured, neutralised and transferred to a nylon membrane (Magnacharge, Genetic Research Instrumentation, Braintree, UK) where it was fixed by ultra-violet (UV) cross-linking. Minisatellite probes were random prime labelled using a BioPrime DNA labelling system (Invitrogen, Paisley, UK) and hybridised to the immobilised DNA/nylon membrane during an overnight incubation at 42°C. Detection and visualisation of the resulting DNA bands was achieved using a KPL Detector AP Chemiluminescent Blotting Kit (Insight Biotechnology Ltd, Wembley, UK) and exposing the membranes to Hyperfilm (Amersham Biosciences, Little Chalfont, UK) for exposure times of 5 min to 6 h. Blots were independently scored by two different assessors and also digitally using Phoretix 1D software (Non-Linear Dynamics, Newcastle upon Tyne, UK) using a 1 kb ladder (Promega, Southampton, UK) for size reference across the well resolved 1–23 kb region. Following exposure, membranes were
stripped of the hybridised probe in 1 l of boiling 0.1% sodium dodecyl sulfate (SDS) (Sigma-Aldrich) and stored in 0.03M sodium citrate, 0.3M sodium chloride, pH 7 (2 × SSC) (Sigma-Aldrich). Criteria for identification of mutations were taken from previously published studies (Dubrova et al 1996, Kiuru et al 2003), i.e. a mutation was considered to be a band present in the offspring that was inconsistent with bands from either parent, and was larger or smaller than the parental band by at least one band-width. Any suspected small mutations were run on a second gel for a longer time period to resolve the size difference between parental and offspring bands.

2.5. Statistical analysis

Mutation rates were calculated for each locus by dividing the number of mutant bands by the number of alleles analysed. The total mutation rate for eight loci was calculated by dividing the total number of mutations by the total alleles analysed. Mean mutation rate was calculated by adding together individual mutation rates for each locus, and then dividing by the total number of loci analysed. Individual locus and total mutation rates were compared using Fisher’s exact test (two-tailed). The distribution of mutations amongst individuals was investigated for evidence of non-randomness against a Poisson distribution using a \( \chi^2 \) test. The distribution of offspring carrying mutations within families was tested for conformity to a binomial distribution using a \( \chi^2 \) test.

3. Results

Data on families of controls and exposed workers are presented in table 1. Mean parental ages at birth of offspring were similar for the control and exposed parents and the proportion of paternal smokers was also similar for the two groups. Data on maternal smoking was not collected since the primary reason for this study was to examine the influence of paternal preconception radiation exposure. Division of the offspring of exposed workers into two dose groups also revealed similar parental age and paternal smoking profiles.

Minisatellite mutation results for offspring from control and exposed workers are presented in table 2. In a few families not all loci were informative and this is reflected in differences in the total number of alleles analysed for each loci. In the control group, 49 mutations were identified in 103 offspring. A total of 37 mutations were of paternal origin and 12 of maternal origin, resulting in a paternal mutation rate of 5.0% (37 mutations in 736 alleles) and a maternal mutation rate of 1.6% (12 mutations in 742 alleles). In the total exposed group 85 mutations were found in 152 offspring, 66 of paternal and 19 of maternal origin, resulting in a paternal mutation rate of 5.8% (66 mutations in 1137 alleles) and a maternal rate of 1.7% (19 mutations in 1133 alleles). Comparison of paternal mutation rates for the control fathers and the exposed fathers revealed an overall rate ratio of 1.16 (95% CI 0.76–1.80) and no statistically significant difference between the groups, either overall (\( p = 0.53 \)) or at any single locus (table 2). A mutation rate of 1.6% was observed for the exposed mothers (12 mutations in 742 alleles) compared with a mutation rate of 1.7% (19 mutations in 1133 alleles) for the unexposed mothers (table 2). The overall rate ratio was 1.04 (95% CI 0.48–2.28) and again, there was no significant difference between the two groups either overall (\( p = 1.00 \)) or at any single locus (table 2).

The 152 offspring of exposed fathers were divided into two dose groups of roughly equal numbers, with doses of 50–175 and >175 mSv, and the minisatellite mutation rates for the two groups were compared with that in the control offspring (table 2). Comparisons of the total
paternal mutation rates of 6.0% and 5.7% with that of 5.0% for the control group revealed rate ratios of 1.20 (95% CI 0.72–2.00) and 1.13 (95% CI 0.68–1.88) respectively and no significant differences ($p = 0.53$ and $p = 0.63$ respectively). The two exposed groups had the same maternal mutation rate of 1.7% and comparisons with the control group mutation rate of 1.6% revealed rate ratios of 1.04 (95% CI 0.39–2.70) and 1.04 (95% CI 0.41–2.60) and no significant differences ($p = 1.00$ for both groups).

Data on the grandchildren are presented in table 3. A total of 23 grandchildren were analysed, 10 with grandfathers in the control group and 13 with grandfathers in the exposed group. Parental age profiles were similar for the two groups. Parental smoking data were not collected. Because most were living locally, checks were made to see if their fathers (i.e. sons and partners of daughters of the control and exposed radiation workers) had a history of occupational radiation exposure. In the event, 11 grandchildren were identified as being associated with some paternal preconceptional exposure, in all but two instances this being <50 mSv. Of the two grandchildren with paternal preconceptional doses >50 mSv, one had a paternal preconceptional dose of 157 mSv and a paternal grandfather in the control group, and the other had a paternal preconceptional dose of 168 mSv and a maternal grandfather in the control group. A total of six mutations were found in the control grandchildren, three of paternal and three of maternal origin, and five mutations in the children of exposed workers, two of paternal and three of maternal origin. Comparison of the paternal rate of 3.9% (three mutations in 77 alleles) in the control grandchildren with that of 2.0% (two mutations in 102 alleles) in the grandchildren of exposed workers revealed a rate ratio of 0.49 (95% CI 0.06–3.75) and no significant difference ($p = 0.65$). Similarly, comparison of the maternal rate of 3.8% (three mutations in 79 alleles) in the control grandchildren with that of 3.0% (three mutations in 99 alleles) in the grandchildren of exposed workers gave a rate ratio of 0.79 (95% CI 0.12–5.08) and the difference was not significant ($p = 1.00$). Comparison of paternal and maternal rates with those of the larger group of control offspring revealed no significant differences for either

| Table 1. Group data for offspring of control and exposed fathers. |
|---------------------------------------------------------------|
| Control group | Total exposed group | Exposed group 1 | Exposed group 2 |
| Number of families | 79 | 97 | 60 | 56 |
| Number of offspring | 103 | 152 | 75 | 77 |
| Mean paternal preconceptional testicular external dose (mSv) (range) | 9 (0–49) | 191 (51–742) | 110 (51–175) | 271 (175–742) |
| Mean paternal preconceptional testicular internal alpha dose (mSv) (range) | <1 (0–3) | 2 (0–56) | 1 (0–56) | 3 (0–44) |
| Mean total preconceptional testicular dose (mSv) (range) | 9 (0–49) | 194 (51–764) | 111 (51–175) | 274 (175–764) |
| Mean paternal age (years) (range) | 28 (19–46) | 31 (23–53) | 30 (23–46) | 33 (24–53) |
| Mean maternal age (years) (range) | 25 (17–37) | 29 (20–41) | 28 (20–41) | 30 (23–40) |
| Number of paternal smokers (%) | 59 (57.3) | 75 (49.3) | 37 (49.3) | 38 (49.3) |

* Some families with more than one offspring appeared in more than one group, this being dependent on father’s external dose at time of conception.

* Maternal age data available for 88 control, 138 exposed, 67 exposed group 1 and 71 exposed group 2 offspring.

* Smoking status at time of conception.
Table 2. Minisatellite mutations detected at eight hypervariable minisatellite loci for different dose groups.

| Locus | Control group | Total exposed group | Exposed group 1 | Exposed group 2 |
|-------|---------------|---------------------|----------------|----------------|
|       | No. of mutations | No. of alleles | Mutation rate (%) | No. of mutations | No. of alleles | Mutation rate (%) | No. of mutations | No. of alleles | Mutation rate (%) | No. of mutations | No. of alleles | Mutation rate (%) |
| Paternal mutations |
| B6.7  | 10 | 82 | 12.2 | 9 | 139 | 6.5 | 0.21 | 7 | 66 | 10.6 | 0.80 | 2 | 73 | 2.7 | 0.04 |
| CEB1  | 13 | 89 | 14.6 | 27 | 136 | 19.9 | 0.37 | 9 | 62 | 14.5 | 1.00 | 18 | 74 | 24.3 | 0.16 |
| CEB15 | 1 | 100 | 1.0 | 3 | 144 | 2.1 | 0.65 | 3 | 71 | 4.2 | 0.31 | 0 | 73 | 0.0 | 1.00 |
| CEB25 | 5 | 100 | 5.0 | 8 | 147 | 5.4 | 1.00 | 5 | 70 | 7.1 | 0.74 | 3 | 77 | 3.9 | 1.00 |
| CEB36 | 2 | 87 | 2.3 | 1 | 138 | 0.7 | 0.56 | 0 | 63 | 0.0 | 0.51 | 1 | 75 | 1.3 | 1.00 |
| MS1   | 3 | 91 | 3.3 | 11 | 141 | 7.8 | 0.26 | 5 | 65 | 7.7 | 0.28 | 6 | 76 | 7.9 | 0.30 |
| MS31  | 2 | 86 | 2.3 | 5 | 141 | 3.5 | 0.71 | 2 | 65 | 3.1 | 1.00 | 3 | 76 | 3.9 | 0.67 |
| MS32  | 1 | 101 | 1.0 | 2 | 151 | 1.3 | 1.00 | 1 | 74 | 1.4 | 1.00 | 1 | 77 | 1.3 | 1.00 |
| Total | 37 | 736 | 5.0 | 66 | 1137 | 5.8 | 0.53 | 32 | 536 | 6.0 | 0.53 | 34 | 601 | 5.7 | 0.63 |
| Mean mutation rate | 5.2 | 5.9 | 6.1 | 5.7 |

| Maternal mutations |
| B6.7  | 1 | 88 | 1.1 | 3 | 132 | 2.3 | 0.65 | 2 | 63 | 3.2 | 0.57 | 1 | 69 | 1.4 | 1.00 |
| CEB1  | 0 | 87 | 0.0 | 1 | 135 | 0.7 | 1.00 | 0 | 63 | 0.0 | 1.00 | 1 | 72 | 1.4 | 0.45 |
| CEB15 | 2 | 98 | 2.0 | 2 | 148 | 1.4 | 0.65 | 1 | 73 | 1.4 | 1.00 | 1 | 75 | 1.3 | 1.00 |
| CEB25 | 1 | 101 | 1.0 | 2 | 145 | 1.4 | 1.00 | 2 | 69 | 2.9 | 0.57 | 0 | 76 | 0.0 | 1.00 |
| CEB36 | 0 | 83 | 0.0 | 3 | 136 | 2.2 | 0.29 | 1 | 64 | 1.6 | 0.44 | 2 | 72 | 2.8 | 0.21 |
| MS1   | 6 | 93 | 6.5 | 6 | 142 | 4.2 | 0.55 | 2 | 65 | 3.1 | 0.47 | 4 | 77 | 5.2 | 1.00 |
| MS31  | 0 | 90 | 0.0 | 0 | 143 | 0.0 | 1.00 | 0 | 66 | 0.0 | 1.00 | 0 | 77 | 0.0 | 1.00 |
| MS32  | 2 | 102 | 2.0 | 2 | 152 | 1.3 | 1.00 | 1 | 75 | 1.3 | 1.00 | 1 | 77 | 1.3 | 1.00 |
| Total | 12 | 742 | 1.6 | 19 | 1133 | 1.7 | 1.00 | 9 | 538 | 1.7 | 1.00 | 10 | 595 | 1.7 | 1.00 |
| Mean mutation rate | 1.6 | 1.7 | 1.7 | 1.7 |

Note: p = probability Fisher’s exact test, two-tailed.
the control grandchildren ($p = 1.00$ and $p = 0.17$ respectively) or the grandchildren of exposed workers ($p = 0.21$ and $p = 0.41$ respectively).

The distribution of paternal and maternal mutations between individual offspring and grandchildren is presented in table 4. For paternal mutations no deviation from Poisson expectations was found for the whole offspring group ($p = 0.948$) nor was there any deviation from expectations in the distribution of paternal mutations in offspring of control fathers ($p = 0.904$) or exposed fathers ($p = 0.983$). Similarly, maternal mutations in the offspring group as a whole were distributed according to Poisson expectations ($p = 0.852$) as were maternal mutations in the control offspring ($p = 0.936$) and offspring of exposed workers ($p = 0.336$). Because of the small numbers, the grandchildren were considered as one single group. Both paternal
and maternal mutations were distributed according to Poisson expectations ($p = 0.949$ and $p = 0.894$ respectively).

Table 5 presents the numbers of children (i.e. offspring and grandchildren) with minisatellite mutations in families with two or more children. Overall a significant deviation from binomial expectations was just reached ($\chi^2_{26} = 12.83, p = 0.046$) for paternal mutations but not for maternal mutations ($\chi^2_{26} = 2.34, p = 0.886$).

In table 6, the mutation data for the total study group is combined to give individual and paternal and maternal rates for the eight loci. A total of 134 mutations were found in 255 offspring and 11 in 23 grandchildren, giving a total of 145 in 278 combined offspring and grandchildren, 108 of paternal and 37 of maternal origin, resulting in a paternal mutation rate of 5.3% (108 mutations in 2052 alleles) and a maternal rate of 1.8% (37 mutations in 2053 alleles).

### 4. Discussion

The suggestion by Gardner et al (1990), that there was a causal relationship between paternal preconceptional radiation exposure and childhood leukaemia, was tested in further epidemiological studies of workers occupationally exposed to radiation. These and other studies of exposed populations, e.g. the Japanese atomic bomb survivors and cancer survivors who have received radiotherapy, have been reviewed by Draper (2008) who concluded that there
has been no confirmation of Gardner’s findings and it seems virtually certain that the increase in childhood leukaemia and NHL that was reported in the vicinity of Sellafield cannot be explained by paternal preconceptional radiation exposure. Despite this increasing epidemiological evidence, the suggestion that radiation-induced instability in the germline, as demonstrated by increases in minisatellite mutations, could provide a mechanism for the cluster of leukaemia near Sellafield continued to be promulgated (Barber and Dubrova 2006). However, the hypothesis that a proportion of childhood leukaemia cases might be associated with an increase in minisatellite germline mutations resulting from parental radiation exposure could not be sustained, when no increase in inherited germline minisatellite mutations was found in children with leukaemia (Davies et al 2007). Indeed, in a recent review, Little et al (2013) conclude that human health has not been significantly affected by such radiation-induced untargeted transgenerational effects.

Initial studies of populations exposed to fallout from the Chernobyl accident (Dubrova et al 1996 1997 2002a) and living in the vicinity of the Semipalatinsk nuclear test site (Dubrova et al 2002b) reported statistically significant increases in germline minisatellite mutations. A further study by the same group also demonstrated a statistically significant increase in the germline mutation rate of exposed fathers living in an area along the Techa River which is contaminated by discharges from the Russian Mayak plutonium facility (Dubrova et al 2006). However, investigations of other exposed populations have not confirmed these findings. Studies of Ukrainian (Livshits et al 2001) and Estonian (Kiuru et al 2003) Chernobyl clean-up workers failed to reveal any significant differences in minisatellite mutation rates in children conceived before the accident compared with children conceived after the accident. Two subsequent studies of Chernobyl clean-up workers, using related techniques, also found no significant increases in germline mutations using multi-locus minisatellite probes (Slebos et al 2004) and microsatellite markers (Furitsu et al 2005). In a study of exposed and control Japanese atomic bomb survivors and their offspring, using the same eight hypervariable minisatellite loci analysed in studies by Dubrova and colleagues, no increase in the germline mutation rate was detected in the exposed families (Kodaira et al 1995 2004). Nor has an increase in microsatellite mutations been observed in the atomic bomb survivors (Kodaira et al 2010). An analysis of sperm DNA from seminoma patients taken both before and after radiotherapy treatment also failed to find an increase in minisatellite mutations (May et al 2000). Similarly, in a study of survivors of childhood and young adult cancer, no evidence was found of an increase in germline minisatellite mutations associated with radiation treatment (Tawn et al 2011). Moreover, in the latter study, detailed analysis of the male cancer survivors in four dose groups revealed no evidence of a dose response, with all dose groups having similar mutation frequencies which did not differ significantly from the control group. In a recent study of residents living in the area of high background radiation in the Kerala region of India, no statistically significant increase in microsatellite or minisatellite mutations was found in comparison with a similar population living in a neighbouring low background radiation area (Ahmad et al 2013).

In a review of available information on the association between radiation exposure and germline minisatellite mutations in humans available at the time, Bouffler et al (2006) noted that those populations with elevated rates were living in areas contaminated with radionuclides where the accuracy of dosimetry and the influence of potential confounders are an issue, whereas populations with predominantly external radiation exposure received acutely or within a short time period, and with well-defined individual dose reconstruction, have not demonstrated raised rates. More recently, Little et al (2013) conclude that there is a weight of evidence that acute high dose paternal exposures have not led to detectable increases of minisatellite mutations in human offspring (Little et al 2013). The situation with regard to
reports of increased minisatellite mutations associated with protracted exposures is less clear and, with no new studies reviewed since Bouffler et al (2006), Little et al (2013) point to the same issues of confounding factors and lack of robust individual dosimetry and highlight the need for further studies of minisatellite mutation in radiation-exposed populations, particularly with internal and/or protracted exposures and where it is possible to minimise confounding factors.

The present study addresses this need and is the first to investigate minisatellite mutations in a population exposed to occupational radiation where the exposure is protracted over many years. Nuclear industry workers have detailed dosimetry records that enable reliable assessments of gonadal doses. In an analysis of mutation rates at the eight most frequently studied hypervariable minisatellite loci, the germline mutation rate of 5.8% observed for the group of men with a mean cumulative paternal preconceptional testicular radiation dose of 194 mSv (range 51–764 mSv) was not significantly different from that of 5.0% for the control group with exposures <50 mSv (mean 9 mSv, range 0–49 mSv) (table 2). Moreover division into two dose groups with means of 111 mSv (range 51–175 mSv) and 274 mSv (range 175–764 mSv) failed to reveal any significant differences and there was no evidence of a dose response (table 2). Indeed, the mutation frequencies observed are similar to those obtained in this laboratory, using the same technique, in a study of mutation rates associated with paternal radiation treatment for childhood and young adult cancer (Tawn et al 2011). In that study, the mutation rate for the male cancer survivors with a mean preconceptional gonadal dose of 1.23 Gy was 5.6% and that of the unexposed male controls was 5.8%.

Studies on tandem repeat mutation rates in mice following paternal F₀ adult radiation exposure have led to suggestions that radiation induces a signal that destabilises the exposed germline. As well as resulting in increased paternal mutations in the F₁ offspring, this instability is also manifest in the F₁ germline thus resulting in a continuing elevation of mutation rate in the subsequent F₂ generation (Barber et al 2002, Barber and Dubrova 2006). Moreover, both male and female F₁ offspring born to irradiated F₀ males showed similarly elevated mutation rates in their germlines. In other words, there is no difference in the transmission of instability through the male or female germline, and mutation rates remain high in the F₂ offspring irrespective of the sex of the F₁ parent with paternal exposure. That being the case, the grandchildren (F₂ generation) of radiation workers in the present study have been considered as a single group and no distinction was made between those born to daughters or sons of radiation workers (table 3). In the event, no significant increases in paternal or maternal mutation rates were observed when the grandchildren from control and exposed grandfathers were compared nor when the two groups of grandchildren were compared to the larger offspring control group, a not unexpected finding since no increase had been observed in their parents. However, numbers of grandchildren were small, with only a total of 23, and are presented here for completeness rather than for firm conclusions to be drawn from the results.

Since radiation-induced minisatellite mutations occur at higher frequencies than expected in comparison with conventional gene mutations, it has been proposed that minisatellite mutation induction is not a targeted event, but rather an untargeted event associated with radiation-induced genomic instability (Niwa and Kominami 2001, Dubrova 2003b, Bouffler et al 2006, Little et al 2013). Mouse studies have shown considerable inter-strain variation in the induction of transgenerational genomic instability (Barber et al 2002) and studies of radiation-induced chromosomal instability in human bone marrow have shown an absence of effect in some individuals (Kadhim et al 1994). If, as suggested, genetic predisposition is an important factor in determining the expression of instability (Wright 2000) then some individuals might be resistant to minisatellite mutation induction whereas others are more susceptible. It might, therefore, be expected that the distribution of mutations between individual offspring
would be skewed, with more individuals having two or more mutations, than if the mutations were distributed according to chance. Consideration of the distribution of paternal and maternal mutations in total offspring and total grandchildren revealed no deviation from Poisson expectations (table 4). Similarly, when the distinction was made between control and exposed workers no deviations in the distribution of paternal and maternal mutations were found for offspring. A previous study in this laboratory reported overdispersion for maternal but not paternal mutations in a study of minisatellite mutations in survivors of childhood and young adult cancer treated with radiotherapy (Tawn et al. 2011). However, the study population and numbers of maternal mutations were small, leading to a cautious interpretation. This previous finding is not supported by the present study which found no suggestion of overdispersion of maternal mutations and thus the present findings are in agreement with those of Kodaira et al. (2004) who found no evidence that paternal or maternal mutations clustered in individual offspring of the atomic bomb survivors.

Genetic predisposition to genomic instability was also investigated by examining the distribution of mutations between families. This enabled the possibility of influences of differences in familial genomic stability to be evaluated. It also provided the opportunity to determine if any specific germline mutations appeared in two or more offspring which would indicate that the mutation had arisen and become established in the germline stem cells. Data from first generation and second generation families with two or more children were combined (table 5). A total of 82 families had two or more children. Fourteen families had two or more offspring who carried paternal mutations whereas no families had two or more offspring with maternal mutations (table 5). In no family was the same mutation seen in more than one offspring. Overall the distribution of offspring with paternal mutations differed marginally from expectations this being driven by the distribution in families with three offspring. For these families, the distribution was skewed with fewer than expected families with all three offspring having no mutations and an excess of families in which all three offspring carried mutations. However, the number of families having three offspring is small and since no evidence of clustering of offspring with mutations was seen in the larger group of families with two offspring it seems likely that this is a chance finding. Moreover, no evidence of familial clustering was observed in a previous study of offspring of cancer survivors who had received radiotherapy (Tawn et al. 2011). The distribution of offspring carrying maternal mutations between families did not deviate from random expectations (table 5). Overall, therefore, this study finds little evidence of familial genomic instability.

Mutation rates at hypervariable minisatellite loci are locus-specific with considerable variation between paternal and maternal events (Vergnaud and Denoeud 2000, Bouffler et al. 2006). For the majority of loci, mutation rate in the paternal is greater than in the maternal germline. Since no significant differences were observed between the different groups in the present study, the mutation rates were combined to give locus and total mutation rates for the overall study group (table 6). The observed differences between loci and between the paternal and maternal germlines were broadly in line with previous reports (Vergnaud and Denoeud 2000, Bouffler et al. 2006). The analysis of 2052 paternal and 2053 maternal alleles is the largest study to date and indicates that for these eight minisatellite loci the total paternal rate of 5.3% is three times that of the maternal rate of 1.8%.

5. Conclusion

This study was initiated at a time of continuing speculation on the role of paternal preconceptional irradiation in the reported increase of leukaemia and NHL in the village of Seascale...
adjacent to the Sellafield nuclear facility. Previous reports of an increase in germline minisatellite mutations in fathers exposed to radiation were seen as a marker of a more generalised radiation-induced genomic instability (Niwa and Kominami 2001) which could provide a mechanistic link between paternal radiation exposure and leukaemia in offspring (Barber et al 2002, Dubrova 2003b). However, the current study found no increase in germline minisatellite mutation rate associated with paternal preconceptional occupational radiation exposure and, therefore, provides concrete evidence against suggestions that such exposure could result in a destabilisation of the germline that can be passed on to future generations.

Acknowledgments

We gratefully acknowledge the support for this study given by the Sellafield workers and their families and thank them for providing samples. The study was facilitated by Dr David Macgregor. Kelly Johnstone, Laura Guyatt, Laura Carter, Cheryl Leith, Shirly Sieh and Michael Trikic contributed to the laboratory analysis. Les Scott, in particular, assisted with provision of dosimetry data from the BNFL worker epidemiology database. We also thank Professor Richard Wakeford whose ongoing support has enabled the completion of this work and who has provided useful comments on the manuscript. The work was supported by the former British Nuclear Fuels plc, the Nuclear Decommissioning Authority and Public Health England’s Centre for Radiation, Chemical and Environmental Hazards.

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