

## Hereditary Minisatellite Mutations among the Offspring of Estonian Chernobyl Cleanup Workers

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A single accidental event such as the fallout released from the Chernobyl reactor in 1986 can expose millions of people to non-natural environmental radiation. Ionizing radiation increases the frequency of germline mutations in experimental studies, but the genetic effects of radiation in humans remain largely undefined. To evaluate the hereditary effects of low radiation doses, we compared the minisatellite mutation rates of 155 children born to Estonian Chernobyl cleanup workers after the accident with those of their siblings born prior to it. All together, 94 *de novo* paternal minisatellite mutations were found at eight tested loci (52 and 42 mutants among children born after and before the accident, respectively). The minisatellite mutation rate was nonsignificantly increased among children born after the accident (0.042 compared to 0.036, OR 1.33, 95% CI 0.80–2.20). Furthermore, there was some indication of an increased mutation rate among offspring born after the accident to workers who had received doses of 20 cSv or above compared with their siblings born before the accident (OR 3.0, 95% CI 0.97–9.30). The mutation rate was not associated with the father's age (OR 1.04, 95% CI 0.94–1.15) or the sex of the child (OR 0.95, 95% CI 0.50–1.79). Our results are consistent with both no effect of radiation on minisatellite mutations and a slight increase at dose levels exceeding 20 cSv. © 2003 by Radiation Research Society

### INTRODUCTION

The accident at the Chernobyl nuclear power plant was the most serious nuclear accident in the world, and it resulted in the release of large amounts of radioactivity into the atmosphere. Despite numerous animal studies, data on

the genetic effects of low radiation doses in humans remain scarce. Data collected in Hiroshima and Nagasaki from children of atomic bomb survivors using standard monitoring systems have not revealed statistically significant differences between control and exposed families in mutation frequencies (1). The detection of changes in germline mutation rate in human populations is extremely difficult. Because of the very low frequency of spontaneous mutation at most loci, enormous sample sizes are required to detect increases in the mutation rate. However, very high rates of spontaneous germline mutations altering the lengths of minisatellite loci have been found in human populations, thus enabling the detection of induced mutation rates in relatively small sample sizes. Minisatellites are highly variable, generally GC-rich DNA sequences characterized by a variable number of tandem repeats of identical 6–100-bp units. They are found with a relatively high frequency throughout the genome and exhibit a diverse variability due to a high spontaneous mutation rate (2–4) that is orders of magnitude higher than that of most protein-coding genes. In humans, instability at GC-rich minisatellites appears to involve different mutation processes in somatic and germline cells. In the germline, minisatellites mutate by a complex recombination-based process that usually results in gene conversion-like transfer of repeat segments between alleles but that occasionally generates true meiotic recombinants within the repeat array (5–8). This recombinational activity appears to be driven by a highly localized recombination hotspot located just upstream from the unstable end of the tandem repeat array. In contrast, in somatic cells, low-level instability results in simple intra-allelic rearrangements (9, 10). Minisatellite mutations consist primarily (but not exclusively) of gains or losses of one or more repeat units. Increased minisatellite mutation frequency has no effect on the viability. Minisatellites appear to be very sensitive to external mutagens such as ionizing radiation. Conflicting results have been obtained on radiation-induced minisatellite mutations in humans. No increase in minisatellite mutation frequency has been detected among chil-

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dren of atomic bomb survivors (11–13) or among Ukrainian Chernobyl cleanup workers (14), yet a doubling of the minisatellite mutation rate has been reported in areas radioactively contaminated by the Chernobyl accident in Belarus (15, 16) and in the population around the Semipalatinsk nuclear test site in Kazakhstan (17). To evaluate the hereditary effects of low radiation doses, we determined the minisatellite mutation rates among the children of 147 Estonian Chernobyl cleanup workers. Minisatellite mutation rates were compared within a family, i.e. between children born before and after their father was exposed to radiation. The post-Chernobyl children ( $n = 155$ ) were conceived within 33 months of the father's return from Chernobyl. Siblings ( $n = 148$ ) born prior to the accident formed the reference group. Paternity was confirmed using five minisatellite probes, and eight additional hypervariable minisatellite probes were used to screen for minisatellite mutations.

## METHODS

### Cohort

The study was conducted according to the relevant ethical rules of Estonia and Finland. The study plan was approved by the Tallinn Medical Ethic Committee. No personal identification data were distributed to the partners outside Estonia.

The cohort of Estonian cleanup workers consists of 4,832 men who had participated, mostly at ages 20–39 years, in cleanup activities in Chernobyl over a median period of 3 months between 1986 (60.4% of workers) and 1991. Identification of this cohort and detailed questionnaire data concerning exposure conditions, dose estimation, etc. have been published (18). While working in the area, the men received a mean ( $\pm$  SD) radiation dose of  $11 \pm 6$  cSv, with less than 1.4% of the cohort receiving more than 25 cSv. The estimate of the men's individual radiation doses was obtained from the database of the Estonian Study of Chernobyl Cleanup Workers; this dose is a physical dose based primarily on thermoluminescence dosimeter (TLD) readings, which gives an estimate of the external whole-body dose from  $\gamma$  radiation. The estimated radiation dose is considered to represent external radiation exposure reflecting the gonadal dose.

### Families

We studied Estonian families in which the father had been a cleanup worker at Chernobyl. An invitation to participate in this study was sent to those Estonian cleanup workers ( $n = 208$ ) who had at least one child born before Chernobyl (pre-Chernobyl child) and at least one child born within 33 months after the father was exposed to radiation at Chernobyl (post-Chernobyl child). All children were born to relatively young parents and were well matched, since the genetic and environmental backgrounds were identical except for paternal exposure to radiation. To have an estimate of the time of conception, and thus of paternal preconceptional irradiation (the stage of spermatogenesis during which the father was exposed to radiation), the gestation age at birth was assumed to be 40 weeks.

### Blood Samples and DNA Extraction

Blood samples were collected from 192 families (father, mother, 198 post- and 148 pre-Chernobyl children) in which the father had been a cleanup worker at Chernobyl. Approximately 20 ml of blood from each donor was collected into sterile Vacutainers and maintained at ambient temperature until delivery to STUK-Radiation and Nuclear Safety Authority in Helsinki, Finland. All samples were received within 24 h after

venipuncture. Genomic DNA was purified from freshly isolated blood lymphocytes using a QIAmp Tissue Kit (Qiagen) and resuspended in  $1 \times$  TE buffer.

### Ascertainment of Paternity

Biological paternity of all children was ascertained by using a PCR-based method where five minisatellite loci (chosen for their low background mutation frequency): APOB (2p23-p24), HRAS1 (11p15.5), MCT118 (D1S80), MCOB19 (D19S20) and YNZ22 (D17S5) were amplified using primers labeled with fluorescent dyes (HEX, 6-FAM, TAM-RA, 6-FAM and HEX, respectively). PCR conditions were optimized for each minisatellite locus, and PCR products were run through 4.5% or 6% acrylamide gels and analyzed with the GeneScan program (Applied Biosystems). The paternity was considered to be false if three or more of the five minisatellite loci used showed a child to have an allele that was not either of the father's alleles on these loci. After incomplete families and families in which the paternity of each child could not be confirmed were excluded, DNA samples from 597 persons from 147 Estonian families (147 father, 147 mother, 155 pre- and 148 post-Chernobyl children) composed the final study material.

### Minisatellite Genotyping

DNA samples (4  $\mu$ g) were digested with *AluI* (Promega) and run through 0.8% (w/v) agarose gels (Agarose NA) in  $1 \times$  TBE buffer (89 mM Tris-borate, pH 8.3, 2 mM EDTA) at 40 V for 24 h. The samples were transferred to a nylon membrane (Hybond N<sup>+</sup>) and hybridized to fluorescein-11-dUTP random primed labeled probes attached to an anti-fluorescein antibody conjugate labeled with alkaline phosphatase [probes (loci): CEB1 (D2S90), CEB15 (D1S172), CEB25 (D10S180), CEB36 (D10S473), MS32 (D1S8), and B6.7 (20q13)] or to commercially alkaline phosphatase-labeled single-stranded oligonucleotide probes [probes (loci): MS1 (D1S7), and MS31 (D7S21)] (NICE<sup>TM</sup>, Cellmark Diagnostics). These loci were chosen for their high background mutation frequency, which enables mutation detection using a reasonable number of examined children. Detection was achieved with the alkaline phosphatase, which catalyzed the chemiluminescent reaction with CDP-Star<sup>TM</sup> as a chemiluminescent substrate either by using GeneImages<sup>TM</sup> CDP-Star<sup>TM</sup> detection module (Amersham Pharmacia Biotech) or non-isotopic chemiluminescence enhanced probe system (NICE<sup>TM</sup>, Cellmark Diagnostics). For visualization the filters were placed against Hyperfilm (Amersham) in a light-tight cassette with intensifying screens at room temperature for 10–60 min. After the filters were probed with each probe, they were stripped with 0.1% SDS and kept moist before hybridization using the other probes. The same blots were hybridized with all eight probes. All autoradiographs were scored over the well-resolved regions (1–23 kb) by eye by two independent assessors, and images were also captured as TIFF images and transferred to computer for analysis. The GelWorks1DAdvanced program (Phoretix International) was used to estimate the sizes of the detected alleles on the basis of the sizes of the  $\lambda$ /HindIII markers (Promega) included on all gels. Mutants were identified as novel DNA fragments present in the children that could not be ascribed to either parent, assuming that they were derived from the parental allele closer in size. Only paternal mutations identified in two independent experiments were included in this study.

### Statistical Analysis

Conditional logistic regression methods were used in the data analysis. The outcome event was minisatellite mutation; i.e., children with confirmed minisatellite mutations were regarded as cases and those without mutations as controls. The unit (stratum) used in the analysis was a family, i.e. comparison of minisatellite mutation frequency between siblings born prior to and after the cleanup work in Chernobyl. Hence children born to the same mother and father were compared with each other. Several children from families with multiple pre- or post-Chernobyl children

**TABLE 1**  
**Paternal Minisatellite Mutation Frequency among Children Born before and after the Chernobyl Accident, with Odds Ratio<sup>a</sup> (and 95% CI)**

Probe	Pre-Chernobyl children			Post-Chernobyl children			Odds ratio (95% CI)
	No. of mutations	No. of paternal meioses	Mutation frequency	No. of mutations	No. of paternal meioses	Mutation frequency	
CEB1	15	148	0.1014	21	155	0.1355	1.38 (0.68–2.80)
CEB15	4	148	0.0270	7	155	0.0452	1.65 (0.48–5.68)
CEB25	4	148	0.0270	4	155	0.0258	1.00 (0.25–4.00)
CEB36	2	148	0.0135	2	155	0.0129	0.84 (0.12–6.12)
MS1	4	148	0.0270	6	155	0.0387	1.41 (0.40–5.02)
MS31	1	148	0.0068	2	155	0.0129	2.00 (0.18–22.1)
MS32	2	148	0.0135	1	155	0.0064	0.50 (0.05–5.51)
B6.7	10	146	0.0685	9	153	0.0588	1.00 (0.35–2.85)
Total	42	1182	0.0355	52	1238	0.0420	1.33 (0.80–2.20)

<sup>a</sup> Odds ratios were obtained using conditional logistic regression analysis of pre- and post-Chernobyl children within a family; i.e., odds ratios based on comparisons between siblings within a family need not correspond to those based on group frequencies.

were included in the analyses if the samples were available (one family with two pre-Chernobyl children and seven with two post-Chernobyl children; of these, only one post-Chernobyl child had a minisatellite mutation). Statistical significance was evaluated using two-sided likelihood ratio tests, and confidence intervals were likelihood-based.

## RESULTS

The paternal origin and change in germline length were determined for 94 *de novo* minisatellite mutations found at the eight tested loci (52 mutants among post-Chernobyl children and 42 among pre-Chernobyl children). The mutation rate was higher in the post-Chernobyl children than in pre-Chernobyl children in four of eight loci, the largest difference being a doubling of mutations at MS31 (Table 1). However, no significant elevation of the mutation rate was seen at any single locus among post-Chernobyl children. The observed minisatellite mutation frequency of all eight single-locus probes combined was slightly higher among the post-Chernobyl children than among the pre-Chernobyl children (0.042 compared to 0.035 per offspring band). In the logistic regression analyses, an odds ratio (OR) of 1.33 (95% CI 0.80–2.20) was obtained.

**TABLE 2**  
**Number of Minisatellite Mutations and Odds Ratios by Interval between Father's Return from Chernobyl and the Date of Birth of the Child**

Interval	Pre-Chernobyl children <sup>a</sup>	Post-Chernobyl children <sup>a</sup>	Odds ratio (95% CI)
38–44 weeks <sup>b</sup>	3/9	1/9	0.33 (0.03–3.20)
45–48 weeks <sup>c</sup>	3/11	3/11	1.00 (0.14–7.10)
49–weeks <sup>d</sup>	28/126	40/133	1.45 (0.84–2.52)

<sup>a</sup> Number of children with minisatellite mutation(s)/total number of children.

<sup>b</sup> Father's mean dose 12.0 cSv.

<sup>c</sup> Father's mean dose 12.6 cSv.

<sup>d</sup> Father's mean dose 12.8 cSv.

No obvious differences were found in the time between the birth of the child and the father's return from Chernobyl (Table 2). Therefore, no adjustment for the father's age or the sex of the child was used in the analyses of dose effects. However, children born more than 49 weeks after the return date had the highest odds ratio (OR 1.45, 95% CI 0.84–2.52).

Possible radiation-induced minisatellite mutations were explored further by subdividing the cleanup workers according to their radiation doses. Children born to fathers with recorded doses of  $\geq 20$  cSv had a statistically nonsignificant increase in the minisatellite mutation rate (OR 3.00, 95% CI 0.97–9.30) relative to their siblings (Table 3). Adjustment for the sex of the child did not alter the results substantially (OR 3.91, 95% CI 0.98–15.6).

The mutation rate was not associated with the father's age (OR 1.04, 95% CI 0.94–1.15), the sex of the child (OR 0.95, 95% CI 0.50–1.79), or the combination of the father's age and the sex of the child (OR 0.84, 95% CI 0.24–2.95).

## DISCUSSION

In this study, the comparison of mutations based on eight hypervariable human minisatellite loci was done between

**TABLE 3**  
**Number of Minisatellite Mutations and Odds Ratios by Father's Estimated Dose**

	Pre-Chernobyl children <sup>a</sup>	Post-Chernobyl children <sup>a</sup>	Odds ratio <sup>b</sup> (95% CI)
4.3–9.9 cSv	16/61	17/66	0.95 (0.44–2.05)
10.0–19.9 cSv	12/48	14/52	1.14 (0.47–2.77)
20.0–30.0 cSv	6/37	13/35	3.00 (0.97–9.30)

<sup>a</sup> Number of children with minisatellite mutation(s)/total number of children.

<sup>b</sup> Odds ratios were obtained using conditional logistic regression analysis of pre- and post-Chernobyl children within a family.

siblings within a family, thus minimizing the variability between exposed and unexposed groups due to genetic and environmental factors. Overall, minisatellite mutations among children born after Chernobyl were slightly yet non-significantly more frequent than among their siblings born before the Chernobyl accident. Children fathered after the accident by men with radiation doses of  $\geq 20$  cSv had a threefold higher rate of minisatellite mutations compared with their siblings born earlier. This result is partly attributable to the fact that the pre-Chernobyl children of the men with the highest doses had a lower minisatellite mutation rate than other groups.

Comparisons with previous studies are limited by differences in radiation exposure. In this study, Estonian Chernobyl cleanup workers received protracted external radiation exposure at a low dose rate over a period of 1–6 months. Individual dose estimates based on personal TLDs was available for two-thirds of the subjects. Biodosimetry results with FISH and GPA methods in this and other cleanup worker cohorts have been consistent with the recorded mean doses (19–21). No meaningful validation of doses at the individual level has been possible due to low doses that approached the minimum level of detectability for biodosimetric methods. Residents of the contaminated areas in Belarus (15, 16) received chronic radiation exposures, both external and internal, with total gonadal doses estimated as roughly 3 cSv. The population around the Semipalatinsk nuclear test site in Kazakhstan was mainly exposed from four surface explosions conducted between 1949 and 1956 with effective doses of over 1 Sv (17). Atomic bomb survivors (1, 11–13) were exposed instantaneously to substantially higher gonadal doses than persons in the Ukrainian (14), Belarussian (15–16), and Estonian studies.

Our results suggesting a slightly yet nonsignificantly increased minisatellite mutation rate for exposed Chernobyl cleanup workers, and especially for those with higher doses, are consistent with an earlier Ukrainian study (14) that shows no excess among exposed workers. This Estonian study and the Ukrainian study (14) are both based on a similar number of subjects as well as on a similar number and type of minisatellite probes used for studying Chernobyl cleanup workers with protracted exposure to low-dose-rate radiation [mean doses of 15 and 11 cSv among Ukrainian (14) and Estonian cleanup workers, respectively]. The background rate of minisatellite mutations was comparable ( $\sim 0.04$ – $0.05$ ) for the unexposed Estonian and Ukrainian (14) populations. The major advantage of our study was a study design using comparisons of exposed and unexposed children within a family; this was not possible in the Ukrainian (14) study, and it may explain the slight difference in results.

Our results are not consistent with those of the Belarussian studies (15, 16) even when only the paternal minisatellite mutations studied using the same eight single-locus probes are considered. The main weakness of the earlier studies (15, 16) was the lack of comparability between ex-

posed and reference groups because the exposed group was from Belarus and the reference population was from the UK. No background rate was available for Dubrova *et al.* (15, 16); i.e., by selecting a control group from UK, they were unable to compare the mutation rate for exposed subjects to one for unexposed subjects from the same population. Since the Belarussian and British populations differ in several ways, the results are prone to the confounding effect of factors other than radiation exposure.

Since our data show only slight differences in the mutation rates between pre- and post-Chernobyl children, it is probable that the minisatellite loci themselves are not the direct targets of radiation. The analysis of mutant alleles at minisatellite MS32 has revealed that the mutation process involves complex conversion-like events and may include gap repair as an important step in the process (5). Radiation may cause DNA damage outside minisatellite loci in the genome. This can result in an increase in DSB repair activity, which could influence the minisatellite mutation rate. It has been shown that mutations at human minisatellites MS32 and MS205 occur predominantly in the germline and most likely at meiosis (5, 6, 10). This raises the question of which stage of human spermatogenesis is most vulnerable to minisatellite mutation induction by radiation. Human spermatogenesis extends over 74 days (22), with a relatively short interval for meiosis. Our results concerning protracted radiation exposure showed that children born more than 49 weeks after the father's exposure to radiation had the highest minisatellite mutation rates, indicating that spermatogonia and stem cells could be most subject to minisatellite mutations. However, our ability to assess the differential sensitivity at various stages of spermatogenesis was limited by the fact that the great majority of offspring were born more than 49 weeks after exposure. The small number of children born during the earlier postexposure period did not allow us to obtain precise estimates for this group. In studies like this Estonian study and the earlier Ukrainian (14) study, drawing conclusions is complicated by the fact that the actual pregnancy week of the child's birth is unknown since it was not included in the questionnaire data. Thus estimations of the stage of spermatogenesis during the father's exposure to radiation are not very accurate, and we must rely on the assumption that all pregnancies are term pregnancies at 40 weeks.

Published animal studies have yielded conflicting results. In mice, an elevated paternal minisatellite mutation rate after irradiation has been found in premeiotic spermatogonia and stem cells (23), at the spermatid stage (24, 25), and at the postmeiotic spermatozoa stage and spermatogonia stage (26).

In conclusion, this Chernobyl study compared minisatellite mutations based on single-locus probes between siblings within a family, thus minimizing the variability due to genetic and environmental factors between exposed and unexposed groups. Overall, this study showed a slight, non-significant increase in the minisatellite mutation frequency

in post-Chernobyl children compared to their pre-Chernobyl siblings. However, there was some indication of an elevated minisatellite mutation rate among children fathered after the accident by men with radiation doses of  $\geq 20$  cSv compared to their pre-accident siblings. The question of low-dose extrapolation is very important in the estimation of the genetic risk of radiation. Although the effect of chance cannot be excluded, our findings provide some evidence for radiation-induced hereditary effects at dose levels exceeding 20 cSv.

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