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\textbf{Research Paper by Drs Dubrova and Jeffreys}

\textit{Attached} is a copy of a research paper by Drs Dubrova and Jeffreys on germline mutation induction for the WG’s consideration.

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Germline mutation induction at tandem repeat DNA loci

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The ability to predict the genetic consequences for humans of exposure to ionising radiation has certainly been as a significant goal of human genetics in the past fifty years. However, despite numerous experimental studies, little is known about the effects of radiation exposure on germline mutation in humans. For example, data collected in Hiroshima and Nagasaki during the past 40 years on children of atomic bomb survivors using standard monitoring systems have not provided evidence of any statistically significant differences in mutation rate between exposed and control families (1). Similarly, a survey of survivors treated with radiotherapy showed that the occurrence of genetic diseases in their offspring was similar to that in control families (2). For this reason, germline mutation induction in mice still remains the main source of experimental data used to evaluate the genetic risk of human exposure to ionising radiation (3,4).

Estimating the genetic hazards of radiation and other mutagens depends on extrapolation from experimental systems. The obvious shortcoming of current approaches for monitoring radiation-induced mutation is the necessity to use very large numbers of individuals (more than 100,000) to detect increases in mutation rate. Furthermore, the question of adequate scoring of radiation-induced mutations by these approaches remains uncertain (5). It is therefore clear that new experimental approaches for monitoring radiation-induced germline mutation in human populations need to be developed.

We have proposed that hypervariable tandem repeat loci may provide a new experimental approach to the evaluation of germline mutation induction in mice and humans by low-dose exposure to ionising radiation (6, 7). These loci have a very high spontaneous mutation rate both in humans (8, 9) and mice (10, 11) and therefore capable of detecting changes in mutation rates in relatively small population samples. Here we present the summary of recent publications analysing mutation induction at mouse and human tandem repeat loci.

MUTATION INDUCTION IN MICE

Tandem repeat loci in the mouse genome include by relatively short microsatellites (<500 bp) with repeat size of 1-4 bp, long expanded simple tandem repeats (ESTR, 0.5-16 kb, repeat size 4-6 bp) and true minisatellites (0.5-10 kb) with repeat size of 14-47 bp (10-14). To date, germline mutation induction has only been studied at the mouse ESTR loci (14-19). These loci were originally termed minisatellites but have recently been renamed to distinguish them from the much more stable true minisatellites in the mouse genome (13, 14). Unstable ESTRs consist of homogenous arrays of relatively short repeats and show a very high spontaneous mutation rate both in germline and somatic cells (10-14), whereas human GC-rich minisatellites have repeat units substantially longer (10-100 bp) and primarily mutate in the germline (20-23). Human minisatellites mutate almost exclusively at meiosis by recombination-based process. Mutation processes at ESTR loci remain unknown though indirect evidence suggests a role for DNA replication/repair in repeat DNA turnover.
To analyse mutation induction, CBA/H male mice were exposed to acute X-rays, chronic \( \gamma \)-rays and high-LET fission neutrons (15, 16, 19). Males were mated to unexposed CBA/H females 3, 6 or 10 weeks post-irradiation, ensuring that litters were derived from irradiated post-meiotic spermatids (3 weeks) or pre-meiotic A, spermatogonia and stem cells (6 and 10 weeks, ref. 24). ESTR mutations in offspring were scored using the mouse-specific single-locus probes MS6-hm and Hm-2 (10, 11). The results of our studies show that:

1. Mutation induction at mouse ESTR loci is suppressed at post-meiotic stages (15, 19).
2. Pre-meiotic acute or chronic exposure to high and low LET ionising radiation causes a statistically significant increase in the paternal mutation rate and a linear dose response, remarkably similar to that for protein-coding genes (15, 16).
3. Statistically significant evidence for mutation induction at ESTR loci was obtained by profiling only 300-400 offspring of control and irradiated parents, whereas traditional systems for monitoring required the analysis of thousands or even hundreds of thousands of mice to detect significant increases in mutation rate.
4. An elevated ESTR mutation rate can be robustly detected at doses that were previously inaccessible using standard approaches for monitoring germline mutation in mice.
5. Mutation induction at ESTR loci cannot be attributed to direct targeted events.
6. ESTR mutation rates in the germline of unexposed first- and second- generation offspring of irradiated male mice are very similar to that in the irradiated F\(_0\) males and unexpectedly do not return to normal unexposed mutation rates (25, Barber \textit{et al.}, submitted).
7. ESTR mutation rates can also be evaluated by a novel single-molecule PCR (SM-PCR) approach, based on the amplification of multiple samples of DNA, each containing approximately one ESTR molecule. SM-PCR allows the detection of indefinitely large number of \textit{de novo} mutants in a single male, does not require profiling the offspring from control and exposed males, and therefore dramatically reduces the numbers of mice needed for the measurement of germline mutation frequencies. Thus, a 1.5-fold radiation-induced increase in germline mutation rate was detected by profiling just 10 exposed and control male mice (26). This also indicates that freshly-formed induced ESTR mutations do appear in the sperm of irradiated males, rather than just pre-implantation lesions.

\textbf{MUTATION INDUCTION IN HUMANS}

Minisatellites include some of the most unstable loci in human genome. In contrast to the mouse ESTRs, they consist of longer repeats (10-60 bp), which show considerable sequence variation along the array (20-23, 27). Mutation at these loci is almost completely restricted to the germline, with very rare and simple mutational events occurring in the somatic cells (20-23). Germline mutation at human minisatellites is attributed to recombination-like events altering repeat unit copy number. Previous studies have shown the very high spontaneous germline mutation rates for some minisatellite loci, ranging from 0.5 up to 13\% per gamete (8, 9), which potentially make these loci useful markers for monitoring germline mutation in humans. The mutational behaviour of human minisatellites is very different from that of mouse ESTRs and the use of mouse ESTR loci as models for human minisatellite instability should be treated with considerable caution.

To date, the frequency of minisatellite mutation has been analysed in families exposed to several different types of ionising radiation (7, 28-31). Mutation rate has also been examined by quantitative small-pool PCR (SP-PCR) methods in human sperm collected from the limited
number of cancer patients exposed to therapeutic mutagens and/or radiation (32-34). These studies have generated conflicting results on the effects of radiation on human germline minisatellite instability. Our data show a statistically significant increase in germline minisatellite mutation rate in two cohorts of irradiated families, namely:

1. **Populations of Belarus and Ukraine exposed to Chernobyl fallout.** Blood samples were collected from 127 full families (father, mother, child) inhabiting the heavily polluted rural areas of the Mogilev district of Belarus and 109 non-irradiated full Caucasian families (father, mother, child) from the UK (7, 29). All families were profiled using multilocus minisatellite probes 33.15 and 33.6 and eight single-locus probes B6.7, CEB1, CEB15, CEB25, CEB36, MS1, MS31 and MS32. Minisatellite mutation rate in the exposed group was found to be two times higher than in the control group. An elevated rate was seen at all three independent sets of minisatellites (detected separately by multi-locus probes 33.15, 33.6 and six single-locus probes), indicating a generalised increase in minisatellite germline mutation rate in the Belarus families. Within the Belarus cohort, mutation rate was significantly greater in families with higher parental radiation dose estimated for chronic external and internal exposure to caesium-137, consistent with radiation induction of germline mutation. Given the fact that the UK control group was of different ethnic origin, the results of this pilot study should be treated with caution. However, we have recently extended the analysis of post-Chernobyl families to another cohort of exposed families from Ukraine (YED, in preparation). In this study, the frequency of minisatellite mutation in children born in the same area before and after the Chernobyl accident was compared. The results of our study again show an elevated mutation rate in the exposed families, therefore confirming the Belarus data.

2. **Population around the Semipalatinsk nuclear test site.** The population around the Semipalatinsk nuclear test site in Kazakhstan provides an unparalleled opportunity for the analysis of the genetic risk of ionising radiation to humans. The Semipalatinsk nuclear test site has been the site for 470 nuclear tests performed by the Soviet Union during the period 1949-89, including atmospheric and surface explosions (1949-63), and underground tests (35). The surrounding population was mainly exposed to the fresh radioactive fallout from four surface explosions conducted between 1949 and 1956, and currently the radioactive contamination outside the test zone is low (35, 36).

Blood samples were collected from 40 three-generation families inhabiting the rural areas of the Semipalatinsk district of Kazakhstan around the Semipalatinsk nuclear test site (30). The control group was composed of 28 three-generation non-irradiated families from the geographically similar rural area of the former Taldy Kurgan district of Kazakhstan, which was not contaminated by nuclear tests. Both groups were matched by ethnicity, year of birth, parental age, occupation and smoking. All families were profiled using eight single-locus probes B6.7, CEB1, CEB15, CEB25, CEB36, MS1, MS31 and MS32.

In the exposed group, a statistically significant 1.8-fold increase in mutation rate was found in the P₀ generation and a less marked 1.5-fold increase was also found in the F₁ generation. All P₀ parents born between 1926-48 were therefore directly exposed to relatively high levels of ionising radiation after these tests. Some F₁ parents (born between 1950-56) were also exposed over this crucial period, whilst those born later were likely to receive considerably smaller doses. This heterogeneity in the parental exposure could explain a relatively moderate 1.5-fold increase in the mutation rate in the F₁ generation. Indeed, germline mutation rate in the
exposed F1 generation shows a negative correlation with the parental year of birth, with the highest mutation rate in the most exposed cohort of parents born before 1960 and similar to that in the P0 families. This negative correlation may therefore reflect the decreased exposure following the decay of radioisotopes in the late 1950’s and after the cessation of surface and atmospheric nuclear tests, thus suggesting that an elevated mutation rate in the affected families is indeed radiation-induced.

Using a similar approach, two another studies do not provide any evidence for radiation-induced mutation in the offspring of irradiated parents (28, 31). This discrepancy could result from totally different types of exposure to ionising radiation in these studies. The atomic bomb survivors from Hiroshima and Nagasaki studied by Kodaira et al. (28) were externally exposed to a considerable dose of acute irradiation. The main exposure to the cohort of Chernobyl clean-up workers was due to external and relatively uniform γ-irradiation, with a relatively minor contribution from the intake of radionuclides (31). In contrast, chronic internal and external exposure was the main source of radiation hazard after the Chernobyl disaster. In addition, most of the children in the Japanese study were born more than ten years after the single acute parental irradiation, which means that at least some radiation-induced DNA alterations could have been repaired over this period of time.

A novel small-pool PCR approach for detection of minisatellite mutations has also been used to quantify germline mutation rate in the cancer patients treated with therapeutic mutagens and radiation (32-34). This approach initially developed for the analysis of spontaneous mutation at human minisatellite loci (20), is based on the amplification of multiple diluted aliquots of sperm DNA and allows the detection of large number of de novo mutants in a single male. Compared to the pedigree approach, this technique dramatically reduces the number of individuals needed for the measurement of germline mutation frequencies. However, the major shortcoming of the SP-PCR approach is a very high variation between spontaneous mutation rates of individual alleles at a single locus (20-23). This variation between alleles may be as high as 10-fold effectively preventing comparisons of mutation rate between non-exposed and exposed men. Therefore, this technique can only be used to evaluate mutation rate in the same man before and after mutagenic treatment. Moreover, SP-PCR does not allow amplification of very large minisatellite alleles (longer than 5 kb), thus restricting mutation scoring to a subset of relatively small alleles.

The analysis of sperm DNA from three seminoma patients before and after radiotherapy has failed to detect any increases in mutation rate at the hypervariable minisatellites B6.7 and CEB1 (33). These men were repeatedly exposed to 15 fractions of acute X-rays with a total testicular dose ranging between 0.4 and 0.8 Gy, a value close to the estimates of doubling dose in male mice (15, 24). The negative results of this study could be attributed to various factors, including the assumption that the yield of germline mutations after fractionated exposure is less than when the same dose is given in a single exposure (37). A direct comparison of the results of this study with our data on the Chernobyl and Semipalatinsk families is limited by the differences in doses and, most importantly, by types of exposure, being acute external for the seminoma patients and chronic internal for the Belarus families.

Conclusions
The results of our studies show the unique advantage of tandem repeat DNA loci for detection of radiation-induced germline mutation in mice and humans. The very high frequencies of spontaneous and induced mutations at mouse ESTRs and human minisatellite loci provide systems capable of detecting induced mutations in relatively small population samples. In mice, an elevated ESTR mutation rate can be robustly detected at doses that were previously inaccessible using standard approaches for monitoring germline mutation, and will provide new insights into the estimation of genetic hazard of low-dose radiation exposure for humans. Given the conflicting results on the effects of radiation on human germline minisatellite instability, more work is clearly needed to validate the potential applications of minisatellite loci for monitoring mutation rate in human populations.

References


