Monitoring spontaneous and induced human mutation by RAPD-PCR: a response to Weinberg *et al.* (2001)

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Weinberg *et al.* (2001, *Proc. R. Soc. Lond. B* **268**, 1001–1005) have recently reported a major increase in mutation rate in the children of Chernobyl liquidators as a result of their fathers' exposure to ionizing radiation. If correct, this would provide dramatic evidence for radiation-induced mutation in humans, and would raise major concerns over the genetic effects of radiation. However, mutants were mainly detected using random amplified polymorphic DNA-PCR, an unreliable technology. These mutants were not validated and had no obvious molecular basis. They may, instead, have arisen as PCR artefacts or through non-paternity or sample mix-up. Unless these mutants can be validated, we recommend that Weinberg *et al.* withdraw their remarkable claims.

Keywords: Chernobyl; liquidator; mutation; radiation; random amplified polymorphic DNA-PCR; validation

Weinberg *et al.* (2001) recently reported a sevenfold increase in mutation rate in the children of Chernobyl liquidators as a result of paternal exposure to ionizing radiation incurred during the clean-up following the disaster. Such an increase is extraordinary, particularly given the fact that most participants in the decontamination work around the Chernobyl nuclear power plant, sarcophagus construction and other clean-up operations received doses of external exposure of less than 0.25 Gy (Pitkevitch *et al.* 1997), well below all estimates of the doubling dose for mammalian germ-line mutation (UNSCEAR 1993). Weinberg *et al.*'s findings also contrast with a recent large study of 183 children of Chernobyl clean-up workers, that showed no evidence for radiation-induced mutation at minisatellite loci (Livshits *et al.* 2001). If Weinberg *et al.*'s claims are correct, they provide the most dramatic evidence to date for radiation-induced heritable mutation in humans, and raise major concerns over the genetic effects of radiation, not only for the families concerned but also more broadly in the radiobiology and radiation-protection communities. However, their report contains a number of inconsistencies and inadequacies that raise serious concerns over their claims.

The principal concern relates to Weinberg *et al.*'s (2001) estimate of the spontaneous mutation rate. They mainly used random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) to detect mutations. This technique uses short random-sequence PCR primers to amplify DNA segments from the human genome (Williams *et al.* 1990). Other than in their earlier study (Weinberg *et al.* 1997), this method has, to our knowledge, never been used to monitor mutation in humans. By comparing parental DNA profiles with profiles from children from unexposed families, they found six new DNA fragments (95% confidence interval (CI), derived from the Poisson distribution, 2–13) among 75 200 bands scored in 50 offspring. They attributed these to spontaneous base substitutions that create new primer sites in

human DNA and, thus, new-length PCR products. This claim appears highly implausible.

The RAPD profiles in Weinberg *et al.*'s (2001) fig. 1 show perhaps 30–40 amplified DNA fragments produced using one 10 nt primer. Though not mentioned, this suggests that about 40 different RAPD primers must have been used in the study to give the 1500 bands scored per child. The spontaneous rate of appearance of new bands is therefore about $6/40/50 = 0.003$ per RAPD test per child (95% CI, 0.001–0.006). The RAPD profiles contained bands up to 3.5 kb in length (Weinberg *et al.* 2001, fig. 1), whereas all new bands were only 200–900 bp long. This implies that many new bands (perhaps 80%) must have been missed due to poor resolution or band superimposition, and that the true mutation rate to new bands could be even higher. However, in the following analysis we shall assume that all mutants have been scored.

The question now is how to convert this rate of appearance of new bands to a spontaneous base mutation rate. Weinberg *et al.*'s (2001) RAPD profile complexity indicates that the effective length of their RAPD-PCR primer must be about 9 nt rather than 10 nt, probably due to mismatches being tolerated at the 5'-end of the primer. The 3×10^9 bp haploid human genome will contain a predicted 11 400 exact matches to a 9 nt sequence ($3 \times 10^9/4^9$), of which about 36 pairs would be close enough (< 3.5 kb) and in the correct relative orientation to produce a RAPD-PCR product in the size range that they see (< 3.5 kb). This number of paired sites is close to the RAPD profile complexity that Weinberg *et al.* observed. A new mutant band would arise by a base mutation at a cryptic site 8/9 bp identical to the effective primer sequence that happens to be correctly orientated and within 3.5 kb of a perfect site. Assuming random site distribution, the genome will contain about 2100 of these paired sites and, thus, 2100 bases at which mutation could be detected. Only one in three mutations at the exact site of mismatch in the cryptic site will create a perfect site. The new-band mutation rate of 0.003 per test observed by Weinberg *et al.* therefore equates to a base

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substitution rate of $0.003 \times 3/2100 = 4.3 \times 10^{-6}$ (95% CI, 1.4×10^{-6} – 8.8×10^{-6}). This estimate is totally at odds with Weinberg *et al.*'s estimate of 4.5×10^{-10} – 1.2×10^{-8} , determined using an unspecified 'computer PCR amplification' procedure.

To check whether this major discrepancy results from errors in estimating paired site frequencies in human DNA, we used an alternative approach that makes no assumption about site distribution. If, as is likely, the complexity of the RAPD profile is at steady state from generation to generation, then the rate of gain of new bands must equal the rate of loss of old bands (such losses could not have been detected in Weinberg *et al.*'s (2001) study). Band loss will result from any base substitution in the 2×9 bp of PCR primer site sequence flanking an amplicon. Since the rate of loss at steady state is equal to the rate of gain, estimated as (six new bands)/(75200 bands) = 8.0×10^{-5} , this implies a spontaneous base mutation rate of $8.0 \times 10^{-5}/18 = 4.4 \times 10^{-6}$ per nucleotide site (95% CI, 1.5×10^{-6} – 9.6×10^{-6}), very similar to our estimate above. These estimates of spontaneous base mutation rate are orders of magnitude higher than the generally accepted estimate of spontaneous human mutation of *ca.* 2×10^{-8} per base per gamete (Nachman & Crowell 2000), and are totally incompatible with levels of nucleotide diversity in the human genome (Cargill *et al.* 1999). We therefore conclude that, despite Weinberg *et al.*'s claim, their new bands cannot be the result of base substitution.

Perhaps these changes are instead the result of insertions and/or deletions between primer sites that are sufficiently large to create a well-resolved mutant. However, 37 bands per test correspond to a target of about 65 kb (37×1.75 kb) being screened for such changes. The observed frequency of 0.003 per child corresponds to 140 such events across the genome, at least as high as the generally accepted incidence of *de novo* base substitutions (Nachman & Crowell 2000). However, such length mutations are known to arise at well below 10% of the frequency of base substitutions (Nachman & Crowell 2000), implying that length mutation cannot be the source of new RAPD bands. Maybe, instead, some of the RAPD bands contain relatively unstable loci. Unstable minisatellites (Jeffreys *et al.* 1988), which account for about 0.03% of the human genome (Knowlton *et al.* 1986; Jeffreys 1987), might be represented in a few of the RAPD products, though Weinberg *et al.* (2001) make no mention of such repeat DNA in the six mutant bands that they sequenced. Microsatellite instability is unlikely to be an explanation, given that most mutation events result in the gain or loss of a single repeat unit (2–4 bp) (Weber & Wong 1993) and could not be resolved in their RAPD profiles. Transposition in the human genome is very rare (Prak & Kazazian 2000) and could not account for the mutants.

So, what could be the explanation for these 'mutants' that appear to have no obvious molecular basis? The trivial possibility is non-paternity or sample mix-up. Surprisingly, Weinberg *et al.* (2001) do not discuss this critical issue nor give any reassurance that the authenticities of samples and relationships have been validated by appropriate testing. Another possibility is variation between DNA samples. For instance, salt contamination could result in additional bands due to mispriming appearing reproducibly on sample retesting. Other

potential problems include somatic DNA rearrangements (acknowledged by Weinberg *et al.*), microbial or viral RAPD products from contaminated or infected blood samples, and carry-over PCR contamination. We note that only two out of the six sequenced 'mutants' showed homology to human/rodent sequences, with no indication that any of them were definitively of human origin.

The detection of *de novo* mutation in the human germ line remains a major scientific challenge and must depend on validated technology coupled to a secure biological base. RAPD-PCR and the 'mutants' detected not only in the unexposed children but also in the exposed families remain unvalidated, with no evidence that these changes genuinely result from primary sequence changes in genomic DNA. Mutant verification is, today, a straightforward task, given the power of sample validation plus the ease of cloning and sequencing mutant bands, determining the relevant locus in human DNA, then re-sequencing parental and offspring DNA to confirm and define the nature of mutation. Unless Weinberg *et al.* can provide such validation evidence, we recommend that they withdraw their extraordinary claims.

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