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Very high mutation rate in offspring of Chernobyl accident liquidators

H. Sh. Weinberg[1], A. B. Korol[1], V. M. Kirzhner[1], A. Avivi[1], T. Fahima[1], Eviatar Nevo[1]*, S. Shapiro[2], G. Rennert[2], O. Piatak[3], E. I. Stepanova[3] and E. Skvarskaja[3]

[1]*Institute of Evolution, University of Haifa, Haifa 31905, Israel*
[2]*National Kupat Holim Cancer Control Center, Carmel Medical Center and Technion Faculty of Medicine, Haifa 34362, Israel*
[3]*Research Center for Radiation Medicine, Academy of Medical Sciences of Ukraine, Melinkova Street 53, Kiev 254050, Ukraine*

Exposure to ionizing radiation has long been suspected to increase mutation load in humans. Nevertheless, such events as atomic bombing seem not to have yielded significant genetic defects. The Chernobyl accident created a different, long-term exposure to radiation. Clean-up teams (or 'liquidators') of the Chernobyl reactor are among those who received the highest doses, presumably in some combination of acute and chronic forms. In this study, children born to liquidator families (currently either in the Ukraine or Israel) conceived after (CA) parental exposure to radiation were screened for the appearance of new fragments using multi-site DNA fingerprinting. Their sibs conceived before (CB) exposure served as critical internal controls, in addition to external controls (non-exposed families). An unexpectedly high (sevenfold) increase in the number of new bands in CA individuals compared with the level seen in controls was recorded. A strong tendency for the number of new bands to decrease with elapsed time between exposure and offspring conception was established for the Ukrainian families. These results indicate that low doses of radiation can induce multiple changes in human germline DNA.

Keywords: germline mutation; Chernobyl; liquidators; DNA fingerprinting

1. INTRODUCTION

The effects of ionizing radiation on somatic cells have been studied using human and mammal subjects, primarily from the viewpoint of radiation-induced cancer risk and dose reconstruction (Akiyama *et al.* 1996; Wishkaerman *et al.* 1997). Although a radiation-induced increase in somatic mutation rate and risk of cancer was documented in a few studies, little is yet known about the impact of ionizing radiation on the mutability of human germ cells. Data obtained using model organisms have shown that ionizing radiation increases the rate of germ cell mutations (Neel & Lewis 1990; Dubrova *et al.* 1993, 1994, 1998; Sadamato *et al.* 1994). In humans, however, it has not been convincingly demonstrated that exposure to low-dose radiation causes heritable changes in germ cells that lead to an increased rate of *de novo* mutations in the progeny. Because of the very low mutation rates per locus, enormous sample sizes are needed to address this question when classical genetic methods are employed. Thus, long-term studies of children born to the survivors of the Hiroshima and Nagasaki bombings have revealed only a slight and non-significant increase in the mutation rate (Kodaria *et al.* 1995; Satoh & Kodaria 1996).

An alternative strategy for simultaneously screening a large number of sites across the genome was adopted in Alec Jeffreys' laboratory (Dubrova *et al.* 1993, 1994, 1996, 1997), based on highly mutable mini-satellite markers. An increased germline mutation rate in families from contaminated areas of Belorussia (now Belarus) was demonstrated (Dubrova *et al.* 1996, 1997), corroborating earlier results obtained by this group on mice (Dubrova *et al.* 1993, 1994). Attempts to reproduce these findings using children of atomic bomb survivors failed to reveal a significant increase in mutation rate (Satoh & Kodaria 1996). Nevertheless, multi-site genome tagging, aimed at increasing the sensitivity of mutation detection, became a preferable method in low-dose-effect studies (Neel 1998). Multi-site tagging was also used in our study, based on a polymerase chain reaction (PCR) with random primers. We therefore believe that the reported results (see also Weinberg *et al.* 1997) are representative of a large part of the genome, although the functional interpretation of these changes does not seem to be easy (Neel 1998).

2. MATERIAL AND METHODS

The radiation exposure following the accident in the Chernobyl nuclear power station in 1986 is considered biologically to have been of a low dose (Dubrova *et al.* 1996). However, certain population groups are assumed to have received higher doses. Foremost among these are people who participated in subsequently cleaning up the reactor, referred to as 'liquidators'. About 700 holders of liquidator certificates currently reside in Israel. The present collaborative study was aimed at evaluating possible genetic changes in offspring of the Chernobyl liquidators, using two samples of families: immigrants to Israel and Ukrainian citizens. Families of liquidators with two or more children, of which one was conceived before (CB) and one after (CA) the exposure of one of the parents to radiation, were tested. In all of the selected families, it was the male parent who was exposed to radiation, with the exception of one Israeli

*Author for correspondence (nevo@research.haifa.ac.il).

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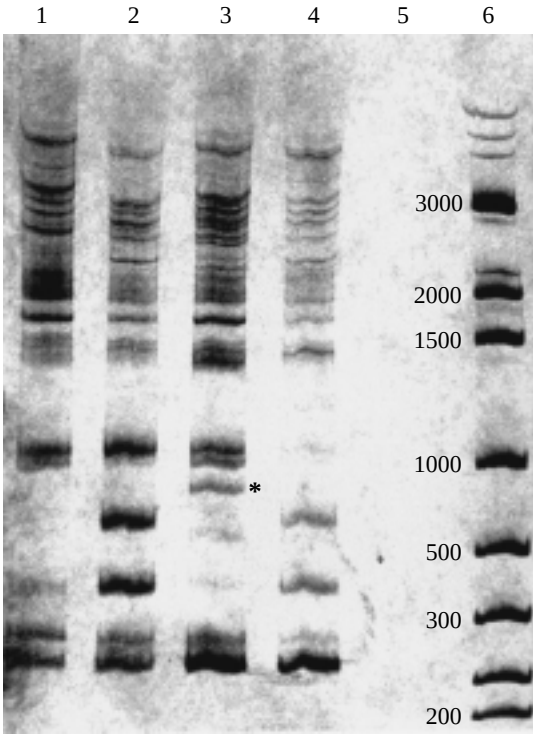

Figure 1. Appearance of a new band in the child conceived after parental exposure. The band marked by a star (size about 750 bp) neither appears in the parents nor in the sib conceived before the accident. The example presented refers to RAPD fingerprinting with primer BC460 (ACTGACCGGC). Lane 1, mother; lane 2, father; lane 3, child conceived after (CA) exposure; lane 4, child conceived before (CB) exposure; lane 5, PCR amplification with no template DNA (negative control); lane 6, pGEM-marker.

family in which both parents were exposed. The majority of liquidators in our study (*ca.* 80%) participated in the cleaning up of the reactor, starting from April–July 1986 (the period of maximum radiation levels). The remaining liquidators started working there in September–October 1986, and only one began in September 1987. In total, 41 CA and 22 CB (internal control) children were studied, whereas the external controls included 28 children from 14 families from uncontaminated regions of the Ukraine and Russia.

Blood was drawn from both parents and available children after the parents had signed an informed consent form, which explained the nature and targets of the study. Blood samples were stored at $-80\,^{\circ}$C until DNA was extracted according to the method of John *et al.* (1991). DNA fingerprinting was carried out by two different PCR methods: (i) random amplified polymorphic DNA (RAPD) (Williams *et al.* 1990; Singh & Roy 1999; Jones & Kortenkamp 2000), and (ii) inter-simple sequence repeats-PCR (inter-SSR-PCR) (Zietkiewicz *et al.* 1994). PCR products were separated by polyacrylamide gel electrophoresis (PAGE) and silver staining (SS). For each marker–family combination where a non-parental band was detected, the entire experiment was repeated three times to ensure reproducibility of the result. The frequency of new amplified bands in the children's DNA fingerprints not appearing in the parents' was evaluated. Each of these changes was tested for reproducibility in three independent PCR runs. Other characteristics like age, sex, time between parental exposure and conception, etc., were also registered. Log-linear models, nonlinear least-squares approximations, and non-parametric tests were employed in data analysis.

3. RESULTS

New DNA fragments (size range 200–900 bp) were detected in CA children as compared with the parents' banding pattern (figure 1). The frequency of appearance of these *new bands* fits the Poisson distribution well (not shown), pointing to independence of the mutation events in the genome. More than a sevenfold increase in the frequency of new bands not present in either of the parents was revealed in CA children, as compared with their CB sibs and controls (difference significant at $p < 10^{-6}$, table 1). Neither marker type (RAPD versus inter-SSR-PCR) nor locality (Israel versus the Ukraine) had a significant effect on the proportion of new bands in the total number of bands recorded in offspring. The same conclusion holds for the absolute number of new bands: neither of the interactions 'marker' × 'birth' or 'place' × 'birth' appeared to be significant.

Six new bands (length range of 250–740 bp) were cloned in p-GEM T-easy vector (Promega, Madison, WI, USA) and sequenced. Remarkably, two of the cloned and sequenced bands showed highly significant homology to some sequences from the world DNA database. One sequence manifested significant similarity to mouse and human *c-fes/fps* proto-oncogene: $p < 10^{-10}$ and $p < 10^{-4}$, respectively. The second sequence gave a homology with rat mRNA for oncogene-induced transin-2 oncogene ($p < 1.2 \times 10^{-39}$).

In addition to the foregoing two marker systems (RAPD and inter-SSR-PCR), we conducted a pilot test in W. Rohde's laboratory at the Max-Plank Institute at Koln using another class of PCR markers, ISTR (Rohde *et al.* 1995). The revealed pattern was the same: a sevenfold increase in the frequency of new bands as compared with the control level (H. Sh. Weinberg, W. Rhode and E. Nevo, unpublished results).

The data in table 1 point to a highly significant increase in mutation rate relative to the control level as a result of parental exposure to ionizing radiation. We were interested in obtaining an estimate of absolute values of spontaneous and induced rates of mutations, as detected by the PCR technique that we employed. For this purpose a simple model was used, based on the assumption that the amplification of new bands derives from radiation-induced, single base changes, replacements, deletions, and insertions along the template, resulting in matching with the corresponding primer sequence. Using 'computer PCR amplification' for some three million base pair contigs of the human genome (taken from different chromosomes), we first evaluated the Spearman's rank correlation between the observed and expected number of bands for each primer; it appeared to be *ca.* + 0.75. Then, we calculated the expected potential number of sites that could mutate to produce sequences that match with our set of primers, while meeting the condition that the size of the 'amplified' band lies within a range corresponding with our experiment. As a result, an estimate $\mathcal{N} = (0.22/6.0) \times 10^8$ of potential nucleotide sites per genome was obtained (the range depends on the assumptions about the matching conditions between the primer and the

Table 1. *Distribution of new (N) and parental-type (P) bands among children in Israeli and Ukrainian liquidator families conceived before (CB) and after (CA) parental exposure*

(Total (Israel + Ukraine, RAPD + inter-SSR-PCR) for CA versus CB (IC): χ^2 (d.f. = 1) = 40.4, $p < 10^{-6}$. Total (Israel + Ukraine, RAPD + inter-SSR-PCR) for CA versus EC: χ^2 (d.f. = 1) = 94.6, $p < 10^{-6}$. Abbreviations: IC, internal control; EC, external control.)

country	born CB/CA children	number	band N/P	number of bands RAPD	number of bands SSR	test of significance for the proportion of new bands[a]
Israel	CA	23	N	39	15	for CA versus CB (IC)
			P[b]	34.50	15.52	χ^2 (d.f. = 1) = 10.8, $p < 0.002$
	CB (IC)	4	N	0	1	
			P	6.00	2.70	
Ukraine	CA	18	N	28	11	for CA versus CB (IC)
			P	27.00	12.15	χ^2 (d.f. = 1) = 29.6, $p < 10^{-6}$
	CB (IC)	18	N	5	0	
			P	27.00	12.15	
	EC	28	N	1	1	
			P	42.20	18.90	

[a] Based on log-linear analysis.
[b] Instead of P, we provide in the table the value of $P \times 10^{-3}$.

template sequences allowing amplification). Using the observed frequency of 0.27 *de novo* bands per individual in the internal control, we obtained the range of estimates for the spontaneous mutation rate as $p = 0.27/N = 4.510^{-10}/1.2 \times 10^{-8}$. This range fits quite well with other estimates of mutation rate in humans obtained by different methods (e.g. Anagnostopoulos *et al*. 1999; Nachman & Crowell 2000). Correspondingly, taking the experimental value of 2.02 new bands per individual, we can estimate the frequency of induced mutations as $3.4 \times 10^{-9}/9.0 \times 10^{-8}$ per base pair.

4. DISCUSSION

An important question related to the interpretation of the obtained results concerns the source of the new bands revealed: are these a manifestation of somatic changes in the tested children, or did the changes occur in the germline cells of the parent(s) and then become transferred to the next generation via gametes? It would be premature to argue that we have the answer to this question. Indeed, one may assume that the origin of the registered changes is somatic mutation in the CA children. But, if so, how can one explain the much lower frequency of such changes in their CB sibs, who were subjected to the same environmental factors during the same or even a longer period? If the environment is the inducing factor, one would expect CB children also to manifest the same or a higher rate of changes. The fact that this is not the case is the basis of our conviction in the germline hypothesis. Therefore, our strategy of internal control, e.g. comparing CA with CB sibs, strongly supports the conclusion that the new bands are indeed a manifestation of changes (presumably point mutations) in the germline cells.

Further strong support for the germline hypothesis in explaining the new bands comes from fitting the observed number of new bands per child as a regression function of the main epidemiological and biological parameters. These included: location (Israel or the Ukraine); period between exposure and conception of CA children; duration of the liquidators' work at the contaminated region; and age of the parent(s) and their children. It is interesting that, for the Ukrainian families, the fitted regression model constitutes a significant explanation ($p < 10^{-2}$ to 10^{-5}) of up to 75% of individual variation in the number of new bands (figure 2). A strong trend was established from this evidence for a decrease in the number of new bands with time elapsed between radiation exposure and conception of CA children (figure 2*c*). An additional explanatory factor included in the models was the *duration* of the liquidators' work in the contaminated region. A decrease in the somatic mutational effect of radiation with time is already known (Pomerantsova *et al*. 1976, 1996, 1997; Alvantic & Searle 1985; Shea & Little 1997), presumably due to a purifying selection in cell populations within an exposed individual. Germline selection in model organisms is an established phenomenon that may be of general importance in the evolution of higher organisms (Hastings 1991; Otto & Hastings 1998). However, we are not aware of any relevant evidence of such an effect in humans, especially in the context of low-dose radiation. Although our results for Israeli families show the same trend, the correlation between the number of new bands and the explanatory variables was much lower. Still, for the combined data (the Ukrainian and Israeli families), the correlation was significant for RAPD ($p < 0.03$) and RAPD + inter-SSR-PCR ($p < 0.01$) markers. A hypothetical explanation for the higher frequency of new bands in the offspring born after parental exposure could be the potential effect of parental age at conception (since the parents were younger at the conception of siblings born before exposure) (for a review, see Crow 1997). If this was a real factor in the observed sevenfold increase in mutation rate, then one would observe a highly significant effect of parental age in both cohorts, as well as in the external controls (families from a non-contaminated area). However, neither the liquidators' families nor the external controls showed such a pattern of results.

(*a*)

Approximation of the frequency of new bands as a function of time to conception and exposure duration

data set	sign of the effect of variable: conception	sign of the effect of variable: duration	R^2 (%)	p
		RAPD		
Israel	–	–	15.1	n.s.
Ukraine	–	+	63.2	0.001
total	–	+	25.8	0.03
		SSR		
Israel	–	–	10.9	n.s.
Ukraine	–	+	59.3	0.01
total	–	+	21.6	n.s.
		RAPD + SSR		
Israel	–	–	17.7	n.s.
Ukraine	–	+	78.6	1×10^{-5}
total	–	+	32.0	0.01

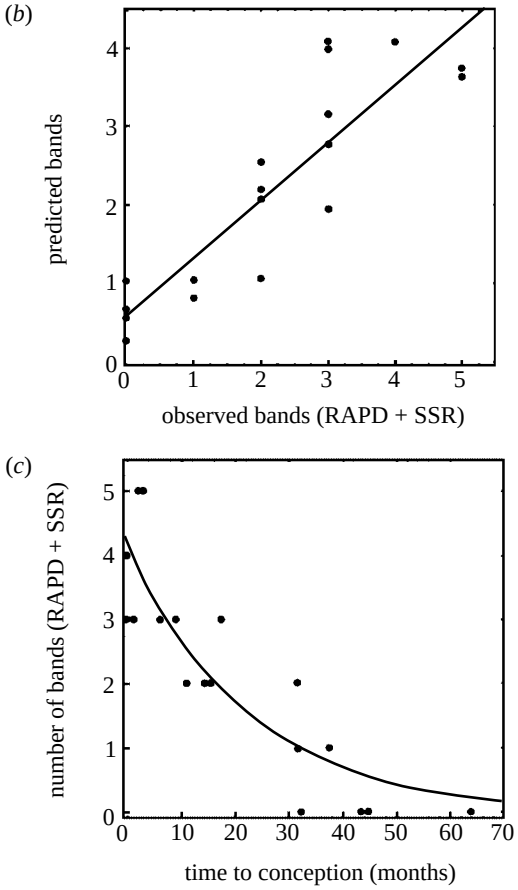

Figure 2. Inter-individual variation in the number of new bands explained by a few epidemiological variables: (*a*) the sign of the effects (minus or plus) and the proportion of the explained variation (R^2); (*b*) the relationship between the observed and predicted number of bands for the Ukrainian data set; (*c*) the total number of new bands (RAPD + inter-SSR-PCR) in children of Ukrainian families and its approximation as a function of the time between parental exposure to radiation and conception. Variables (in the equations below): Loc, location (1, Israel; 2, the Ukraine, so that a plus in the table means a higher number of new bands in CA children of Ukrainian families and a minus means an opposite trend); Conc, time between exposure and conception; Dur, duration of the parental work on the contaminated territory during work as a liquidator. The observed number of bands was approximated, based on minimum least-squares analysis, by a nonlinear regression for the combined data set, $y = b_0\exp((-a_1\mathrm{Conc}\times a_2\mathrm{Conc})\times(\mathrm{Loc}-a_3\mathrm{Dur}))$, and a nonlinear regression for data on families within each country, $y = b_0\exp(-a_1\mathrm{Conc}-a_2\mathrm{Dur})$. In (*c*), the dependence on time between parental exposure and conception was approximated as $y = b_0\exp(-a_1\mathrm{Conc})$. Remarkably, these simple approximations appeared to explain a significant proportion (up to 70–80%) of variation between the children (conceived after parental exposure) in the number of new bands in their fingerprints.

Our results support the conclusions reached by other groups using different methods, demonstrating that low-dose ionizing radiation induces mutational changes in human genome (Nigel 1995; Dubrova *et al*. 1996, 1997) as well as in model organisms (Dubrova *et al*. 1993, 1994; Ellegren *et al*. 1997). According to several attempts at dose reconstruction for Chernobyl liquidators, the modal doses may lie within the interval 50–200 mGy (e.g. Goldman 1997). Unfortunately, no reliable dose reconstruction was possible in our material, so this parameter is not considered in the foregoing analysis. Long-term fundamental studies of mutation rate in the progeny of survivors from the atomic bombing of Hiroshima and Nagasaki have resulted in the estimates of 1–2 Gy of doubling dose for cytogenetic criteria (chromosome aberrations) and point mutations affecting enzyme activity or malformations (Neel *et al*. 1989, 1990; Chekovich *et al*. 1993). Our finding of a greater than sevenfold increase in the frequency of new bands, combined with the above-dose interval, suggests that doses much lower than 50–200 mGy may double the output of *de novo* genomic changes. Without doubt, the answer must depend on the type of changes under study; hence, no real contradiction exists between these two estimates. The first estimate reflects mainly fitness- or health-related changes, whereas the second presumably measures the genomic changes, most of whose consequences may be neutral or nearly neutral for their carriers (tenfold between-locus variation in the rate of radiation-induced mutations was also obtained on experimental models; see Pretsch *et al*. 1994). Nevertheless, the small contribution of these changes to the immediate genetic risk does not exclude the possibility of prolonged effects. These may include induced genetic instability (effects similar to dynamic mutations, or genetic anticipation; Murnane 1996), increased sensitivity to other genotoxic factors or cancer promoters, and change in recombination frequency and distribution (Carole 1998). The very fact that much lower doses of radiation than previously generally believed can double the number of genomic changes needs serious attention. This is all the more important when a significant proportion of the human population is subjected to increased mutagenic pressure due to medical diagnostics, industrial accidents, professional occupation, or general growth in environmental contamination by genotoxic agents.

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