Germ-Line Mutations at a Mouse ESTR (Pc-3) Locus and Human Microsatellite Loci

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Germ-line mutation/ESTR/Microsatellite/Dioxin/Chernobyl.

We examined the use of the mouse Pc-3 ESTR (expanded simple tandem repeat) locus and 72 human microsatellite loci as potentially sensitive biomarkers for mutagenic exposures to germ cells in mice and humans respectively. In the mouse work, we treated male mice with TCDD (2, 3, 7, 8-tetrachlorodibenzo-p-dioxin; a chemical known to induce congenital anomalies in humans and mice) and analysed the F1 fetuses for Pc-3 mutations. Although the incidence of anomalies was higher in the TCDD group, there were no induced mutations. However, respiratory distress syndrome (RDS) was observed in 3 of 7 fetuses born to male mice which were treated with TCDD and which showed abnormal length of Pc-3 allele. In the human studies, the children of Chernobyl liquidators were examined for mutations at a total of 72 (31 autosomal, 1 X-linked and 40 Y-linked) microsatellite loci. This study was prompted by earlier findings of increases in microsatellite mutations in barn swallows and wheat in the highly contaminated areas after the Chernobyl accident. We examined 64 liquidator families (70 children) and 66 control families (70 children). However, no increases in mutation rates were found. The estimated mean dose to the liquidators was about 39 mSv and this might be one possible reason why no increases of mutations could be found.

INTRODUCTION

During the past ten years, several studies have been made on the induction of germline mutations using minisatellite loci in humans and ESTRs (expanded simple tandem repeat loci, previously named as minisatelites) loci in mice.13 Additionally, there have been a limited number of studies using microsatellite loci.2,3 These three types of repeats differ in repeat size and length. For example, the human minisatelites consist of 10 to 60 bp repeats and may span from about 0.5 kb to several kbs. The mouse ESTRs consist of 4 to 6 bp repeats and may span from 0.5 to 16 kb. Microsatellites, also referred to as short tandem repeats are composed of tandemly repeated stretches of short (1 to 6 bp) motifs and can be up to 600 bp long. The main reasons for using these loci in mutagenesis studies are that some of them are highly unstable i.e., show very high spontaneous mutation rates (orders of magnitude higher than protein coding loci) and mutations can also be induced in them. Therefore, changes in mutation rate can be detected at substantially small sample sizes than those required with conventional mutation studies and have been considered to be sensitive biomarkers to study germ line mutations.1,4 The mutational changes are manifest as alterations in the number of tandem repeat cores, and hence, allele length. Two techniques are available for mutation detection, depending on the length of the loci: Southern blot analysis using restriction enzymes, and PCR-based agarose gel or capillary electrophoresis.

In this paper we present the results of our studies on the induction of germ cell mutations (a) at the mouse Pc-3 locus (which belongs to the ESTR family of repeats) in the F1 progeny of male mice treated with dioxin (TCDD: 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin ) and their potential correlation with TCDD-induced congenital malformations and (b) at 72 microsatellite loci in the children to Chernobyl clean-up workers exposed to radiation.

RESULTS

Congenital malformations in F1 offspring of male mice treated with dioxin 2,3,7,8-tetrachlorodibenzo-p-dioxin)

In the first set of experiments, C3H/HeJ male mice were treated intra-peritoneally with TCDD at doses of 50 and 100 ng/g bw and mated with untreated C57BL/6J females. Post-meiotic male germ cells were sampled. Corn-oil treated
males served as controls. Pregnant females were examined in utero to ascertain rates of preimplantation losses, early and late embryonic deaths and the proportion of normal and malformed fetuses (and among the latter those affected by the respiratory distress syndrome (RDS)). The data are summarized in Table 1 which shows that (a) the implantation rates are lower after TCDD treatment (and significantly so after the 100 ng/g bw dose); (b) in both treatment groups, the frequencies of malformations as well as RDS are higher than controls. To confirm and extend the above results, in the second set of experiments, mice from the C.B17 strain was used. C.B17 males were treated with TCDD with 100ng/g bw (spermatogonial stage) by oral intubation and mated with untreated females with same strain. The data presented in Table 2 show again that paternal TCDD treatment (a) adversely affects the implantation rate as well as the proportion of living fetuses and (b) results in significantly higher frequencies of congenital malformations as well as RDS in the fetuses.

The normal and malformed fetuses were used as experimental material to study mutations at the Pc-3 ESTR locus and the results are discussed in the next section.

**TCDD-induced mutations at the Pc-3 locus studied in F1 fetuses of treated males and their potential correlation with congenital malformations**

For studying mutations at the Pc-3 locus, DNAs from the same F1 fetuses (from TCDD treated males discussed earlier) were examined using the technique of PCR- based agarose gel electrophoresis. The reason for this is that the PCR products of both C57BL/6J and C3H/HeJ strains were ~ 1.5 kb (Fig. 1). The results showed that (a) the frequencies of paternal band shifts were 22.2% (16/72), 17.7% (11/62) and 15.1% (8/52), respectively in the controls, 50 ng/g and 100 ng/g TCDD treated groups; (b) the frequencies of maternal band shifts were 4.2% (3/72), 8.1% (5/62) and 7.5% (4/53) respectively, for the control, 50 ng/g and 100 ng/g of TCDD treated groups and (c) the frequencies of maternal Pc-3 mutations in TCDD treated groups was higher than in the controls, but not significantly so. As will be evident, there is no clear correlation between Pc-3 mutations and congenital malformations. Assuming that this is presumably because of small sample sizes, we conducted the next set of Pc-3 mutation experiments with C.B17 mice discussed below. (The results of in utero analysis for these were presented in Table 1.

### Table 1. Incidence of preimplantation loss, embryonic deaths, respiratory distress syndrome (RDS), and malformations in the F1 fetuses C57BL/6J and C3H/HeJ mice treated with TCDD

| TCDD dose (ng/g) | No. of pregnant mice | No. of corpora lutea (Av.) | No. of implant (%) | No. of early death (%) | No. of late death (%) | No. of living fetus (%) | No. of RDS (%) | No. of malformed Fetus (%) |
|------------------|----------------------|---------------------------|-------------------|-----------------------|----------------------|------------------------|--------------|---------------------------|
| 100              | 10                   | 94 (9.4)                  | 73 (77.7)         | 20 (27.4)             | 6 (8.2)              | 47 (64.4)              | 9 (19.2)     | 5 (10.6)                  |
| 50               | 11                   | 98 (8.9)                  | 87 (88.8)         | 15 (17.2)             | 10 (11.5)            | 62 (71.3)              | 18 (29.3)    | 5 (8.1)                   |
| 0                | 11                   | 92 (8.4)                  | 85 (92.4)         | 13 (15.3)             | 3 (3.5)              | 69 (81.2)              | 5 (7.2)      | 1 (1.4)                   |

*: the average numbers of corpora lutea per pregnant female; :% of corpora lutea, :% of implants, :% of living fetus, :1 gigantism. *: p < 0.05, **: p < 0.01, ***: p < 0.001. Malformed fetus in dioxin treated group: 2 dwarf + omphalocele, 1 agenesis of the testes, 1 agnathy + cleft palate + kinky tail + PDA, and 1 eyelid defect + general edema for 100 ng/g; 5 dwarf for 50 ng/g. Control group: 1 dwarf.

### Table 2. Incidence of preimplantation loss, embryonic deaths and malformations in the F1 fetuses of dioxin treated CB.17 mice.

| Dioxin dose (ng/g) | No. of pregnant mice (Av.) | No. of corpora lutea (%) | No. of implant (%) | No. of early death (%) | No. of late death (%) | No. of living fetus (%) | No. of RDS (%) | No. of malformed Fetus (%) |
|--------------------|-----------------------------|--------------------------|-------------------|-----------------------|----------------------|------------------------|--------------|---------------------------|
| 100                | 20                          | 196 (9.8)                | 158 (80.6)        | 33 (20.9)             | 8 (5.1)              | 117 (74.1)             | 35 (29.9)    | 14 (12.0)                 |
| 0                  | 17                          | 179 (10.5)               | 165 (92.2)        | 42 (25.5)             | 3 (1.8)              | 120 (72.7)             | 14 (11.7)    | 4 (3.3)                   |

*: the average numbers of corpora lutea; :% of corpora lutea, :% of implants, :% of living fetus. *: p < 0.05, **: p < 0.01, ***: p < 0.001. Malformed fetus in dioxin treated group: 1 omphalocele, 5 dwarfs, 5 short tails, 1 short + kinky tail, 1 syndactyly, and 1 giant 1- toe. Control group: 1 dwarf, 1 dwarf + cleft palate, 1 Meckel’s diverticulum, and 1 open eyelid.

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The reason for using C.B17 strain was that \( P_{c-3} \) locus in this strain is much shorter (~580 bp long) compared to that of C57BL/6J and C3H/HeJ strains.

The numbers of F1 fetuses examined in these experiments were 127 for TCDD treated group and 140 for control group. The PCR products were applied on capillary electrophoresis (ABI PRISM 310, Applied Biosystems, Foster City, CA, USA) and the products were analyzed by fragment analysis software (GeneScan, Applied Biosystems). Mutation detection in C.B17 was possible even though PCR products of \( P_{c-3} \) locus with one base length change (Fig. 1). The results showed that the mean size of \( P_{c-3} \) alleles was the same in TCDD treated and the control groups. However, seven mice of TCDD treated group had allele sizes outside of 99% confidence limit of the allele size range in untreated controls. Only one mouse in the control group had the size outside of the normal range. The difference was not significant, but interestingly, three of the seven mice with \( P_{c-3} \) mutations had congenital malformations.

MICROSATELLITE MUTATIONS IN THE CHILDREN OF CHERNOBYL LIQUIDATORS

Study subjects

After the Chernobyl nuclear power plant accident in April 1986, many civilians and military personnel from the former Soviet Union conducted clean-up operations. They were called “liquidators”. Their operations included: decontamination, construction, transport, security work, and assistance for evacuation. In collaboration with a research group from Belarus, we conducted a study to examine microsatellite mutation rates in 64 liquidators’ and 66 control families. Of 64 liquidator families, 61 children were born to fathers who had been exposed to radiation before conception (preconceptional exposure) and 9 had been conceived before fathers worked as liquidators (postconceptional exposure). Mothers had no history of radiation exposure. The control families were living in non-contaminated areas in Belarus. Sixty nine children from 66 control families were included in the study. The sex ratio and age of the children for both families were nearly matched.

Microsatellite loci

Thirty one autosomal, one X-linked and 40 Y-linked microsatellites loci were selected on the basis of their high mutation rates published in the literature. The microsatellites loci were amplified by PCR using fluorescent-dye-labeled primers and the PCR products were applied on capillary electrophoresis (ABI PRISM 3100, Applied Biosystems). The sizes of PCR products were analyzed by GeneScan. Mutations were determined as PCR product size shift in the comparison of parents and children (Fig. 2).
Comparison of mutation rates in the children of liquidators who had been exposed to radiation preconceptionally with those of the unexposed control group

The data on mutation rates at the microsatellite loci are presented in Table 3. These were calculated as number of mutations per microsatellite locus per offspring. Mutation rates for the autosomal loci were $5.9 \times 10^{-3}$ (11/1852) and $8.5 \times 10^{-3}$ (18/2108) for the exposed and control groups, respectively. For the Y-linked loci, mutation rates were $2.9 \times 10^{-3}$ (4/1392) and $2.1 \times 10^{-3}$ (3/1458) for the exposed and control groups, respectively. No statistical difference between the exposed and control groups for either autosomal or Y-linked loci could be found ($\chi^2 = 0.916$ for autosomal, $\chi^2 = 0.004$ with Yates’ correction for Y-linked loci). No mutations of X-linked locus were detected (Table 3).

Mutation rates estimated for spermatogonial and post-spermatogonial stages exposure

We assumed that (a) post-spermatogonial stages were exposed when the fathers were working as liquidators within three months before the conception date (estimated by back-calculating nine months before the birth date of the children) and (b) spermatogonial stages were exposed when the fathers’ working period was more than three month before conception.

**Table 3.** Microsatellite mutation rates in the children of male liquidators who had been exposed before conception and those of un-exposed control.

| Microsatellite Loci | Control group | Preconceptionally exposed group |
|---------------------|---------------|---------------------------------|
|                     | No. of mutations (mutation rate, $\times 10^{-3}$) | Total no. of microsatellite loci examined | No. of mutations (mutation rate, $\times 10^{-3}$) | Total no. of microsatellite loci examined |
| Autosomal           | 18 (8.5)      | 2108                            | 11 (5.9)$^a$                                       |
| X-linked            | 0             | 68                              | 0                                                 |
| Y-linked            | 3 (2.1)       | 1458                            | 4 (2.9)$^b$                                       |

$^a$: $\chi^2 = 0.916$  $^b$: $\chi^2 = 0.004$ with Yates’ correction
the estimated conception date. Consequently, spermatogonial exposure is included partly in the group of post-spermatogonial exposure. For the autosomal microsatellite loci, spermatogonial exposure resulted in slightly higher mutation rates $6.7 \times 10^{-3}$ ($7/1046$) than post-spermatogonial stage exposure $5.0 \times 10^{-3}$ ($4/806$), but the difference was not statistically significant ($\chi^2 = 0.031$ with Yates’ correction). For the Y-linked loci, mutation rates were $3.1 \times 10^{-3}$ ($2/653$) and $2.7 \times 10^{-3}$ ($2/739$) for the spermatogonial and post-spermatogonial exposure, respectively. Again there was no difference between two stages ($\chi^2 = 0.143$ with Yates’ correction).

**Nature of mutations detected**

All the microsatellite mutations (except one) that we found involved the gain or loss of one repeat unit. These results and those of others have led investigators to assume the replication slippage model for microsatellite mutagenesis. The exception was that there was an additional PCR product in DYS464 (Y-linked locus) in the child of exposed group. For the autosomal loci, 3 of the mutations detected were maternal and 8 were paternal in origin in the exposed group; in the unexposed group, 1 was maternal and 17 were paternal for the autosomal loci. This difference is also not significant ($\chi^2 = 1.190$ with Yates’ correction).

**DISCUSSION: DOES GENOMIC INSTABILITY CAUSE ADVERSE EFFECTS?**

**Congenital malformation and the mutations at Pc-3 locus in mice**

The choice of TCDD for our studies was determined by earlier report in which TCDD had been found to induce congenital anomalies in man. Our study also showed that incidence of malformation increased significantly in F1 offspring of TCDD treated mice. There was no significant difference in Pc-3 mutation rates between the TCDD treated and the control groups in B6C3 F1 mice. One reason for the lack of a difference might be the high frequency of mutations in the untreated control group as ascertained by the ordinary electrophoresis assay (Fig. 1). Therefore we investigated Pc-3 mutation with high resolution analysis (GeneScan) in C.B17 mice. However, there were no differences in mutation rates between the two groups, while three of seven fetuses that showed abnormal allele size (base length) were found in those fetuses with malformation.

**Spontaneous mutation rates of microsatellites in Belarus and other countries**

The lack of difference in the mutation rates between the children of the liquidators and those of unexposed controls might be due to the higher mutation rates in the unexposed control group (i.e., higher than the spontaneous mutation rates from non-Belarusian populations). In order to examine this, we compared the mutation rate estimates in the unexposed control group with those in other countries reported in the literature (Table 4). Mean spontaneous mutation rates were $3.2 (1.9–5.5, 95\% CI) \times 10^{-3}$ and $5.7 (4.6–7.0, 95\% CI) \times 10^{-3}$ in Belarus, and other countries, respectively, for 22 autosomal loci. For the seven Y-linked microsatellite loci, mean rates were $3.9 (0.6–21.8, 95\% CI) \times 10^{-3}$ and $3.2 (1.9–5.5, 95\% CI) \times 10^{-3}$ in Belarus and, Germany and Poland, respectively. It is thus clear that there are no significant differences in mutation rates between the non-Belarusian countries and our Belarusian control. Furthermore, the mutation rates estimated in our Belarusian control is also consistent with that of any non-Belarusian countries at same set of loci. This suggests Belarusian people might not have been exposed to some chemical mutagens in the environment.

**Estimated radiation dose of the Chernobyl liquidators**

For our study, estimates of radiation doses were not available except for one liquidator whose dose was reported as 1.600mR. According to the UNSCER report (2000), the mean effective dose of the liquidators were 39mSv (1986–1987) in Belarus, 169mSv (1986) and 92mSv (1987) in Russia, and 185mSv (1986) and 112mSv (1987) in Ukraine. Therefore, one of the reasons for the negative effects in our study might be related to the low dose sustained by the Belarusian liquidators.

**Human studies on transgenerational mutations at tandem repeat loci after radiation exposure reported in the literature**

Two studies, which showed “negative” effects on the microsatellite mutations, have been reported. One was a pilot study in which three autosomal and two X-linked microsatellite loci were used in the children of the atomic bomb survivors. Another was the study in the children of the Chernobyl liquidators in Ukraine using five autosomal and one X-linked microsatellite loci. A modest increase in one tetranucleotide repeats (D7S1482) was observed, but this was not significant. Induced mutation frequency of microsatellites was reported to be $8.75 \times 10^{-3}$/locus/Gy/cell in the human glioma cell. Both germ cell studies did not show any definitive effects due to a small number of microsatellite loci were used. On the other hand, significant increases of germ-line mutation rates were reported for the barn swallows and wheat in highly contaminated areas by the Chernobyl accident. In the wheat study, the estimated dose was about 0.3 Gy (total dose of external and internal exposure) of chronic irradiation. It seems worthwhile to undertake studies on the induction of germ-line microsatellite mutation in rodents.

Several studies had been conducted to study germ line effects of radiation using minisatellites loci as bio-markers. The first positive effect was reported in the children of residents in contaminated areas in Belarus, however UK popu-
ulation was used as the unexposed control. Then, later on, the same authors reported that there was significant increase in the F1 offspring of residents in contaminated areas in Ukraine when the children born in the same area before accident were used as unexposed controls. Positive effect was again reported in the children of residents around the nuclear test site in Kazakhstan. On the other hand, the negative effects were reported in the children of the A-bomb survivors and the liquidators in Estonia and Ukraine. The reasons for the discrepancy are not clear. However, one possible reason for the positive effects reported might be that the children of the residents in contaminated areas might be exposed continuously to radiation and chemicals for long time during fetal and post-natal stages, and in addition to preconceptional stage.

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**Table 4.** Spontaneous microsatellite mutation rates for the same set of loci in Belarusian unexposed controls and those in other countries.

| Microsatellite loci | Mutation rates ×10⁻³ (95% confidence interval) | Country name |
|---------------------|-----------------------------------------------|--------------|
|                     | Un-exposed control (Belarus)                  |              |
| CSF1R(CCTT/CTTT), CSF1R(TAGA) | 29.4 (11.3–72.6) | Japan¹     |
| CSF1R(CCTT/CTTT), CSF1R(TAGA) | 29.4 (11.4–72.2) | Finland²    |
| D19S244, D19S245, D19S47, D19S247 | 3.7 (0.7–20.6) | USA³       |
| D13S120             | 14.7 (2.7–79.2) | USA⁴       |
| D18S51, D21S11, D8S1179, FGA, ACTBP2 | 4.9 (1.3–17.7) | Germany⁵   |
| D7S1517             | 14.7 (2.7–79.2) | Germany⁶   |
| D18S51, vWA, FGA, D8S1179, D21S11 | 2.9 (0.5–16.4) | Portugal/Spain⁷ |
| D12S1090            | 29.4 (7.9–100.4) | Belgium⁸   |
| D12S391, vWA, D12S391, D21S11, FGA, ACTBP2 | 5.9 (1.6–21.2) | Germany⁹   |
| D4S2431, D5S2501, D10S1237, D15S57 | 2.9 (0.5–16.4) | Brasil¹⁰   |
| D18S1270            | 2.9 (0.5–16.4) | Brasil¹⁰   |
| Mean                | 3.2 (1.9–5.5) | Mean      |
|                     | 5.7 (4.6–7.0) | Mean      |

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