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# Dose–response curves for analyzing of dicentric chromosomes and chromosome translocations following doses of 1000 mGy or less, based on irradiated peripheral blood samples from five healthy individuals

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## ABSTRACT

In terms of biological dosimetry at the time of radiation exposure, the dicentric chromosome (Dic) assay (DCA) is the gold standard for assessing for the acute phase and chromosome translocation (Tr) analysis is the gold standard for assessing the chronic phase. It is desirable to have individual dose–response curves (DRCs) for each laboratory because the analysis criteria differ between laboratories. We constructed the DRCs for radiation dose estimation (with three methods) using peripheral blood (PB) samples from five healthy individuals. Aliquots were irradiated with one of eight gamma-ray doses (0, 10, 20, 50, 100, 200, 500 or 1000 mGy), then cultured for 48 h. The number of chromosome aberrations (CAs) was analyzed by DCA, using Giemsa staining and centromere-fluorescence *in situ* hybridization (centromere-FISH) and by chromosome painting (chromosome pairs 1, 2 and 4) for Tr analysis. In DCA, there was large variation between individuals in the frequency of Dics formed, and the slopes of the DRCs were different. In Tr analysis, although variation was observed in the frequency of Tr, the slopes of the DRCs were similar after adjusting the background for age. Good correlation between the irradiation dose and the frequency of CAs formed was observed with these three DRCs. However, performing three different biological dosimetry assays simultaneously on PB from five donors nonetheless results in variation in the frequency of CAs formed, especially at doses of 50 mGy or less, highlighting the difficulty of biological dosimetry using these methods. We conclude that it might be difficult to construct universal DRCs.

**Keywords:** dicentric chromosome; chromosome translocation; dose–response curve; biological dosimetry; Giemsa staining; centromere-FISH

## INTRODUCTION

Several methods have been reported for rapid biological dosimetry immediately following exposure to low and high doses of radiation [1–8], of which the most reliable for international standardized biological dosimetry is the dicentric chromosome (Dic) assay (DCA) [9]. DCA is typically used following acute radiation exposure of between 100 mGy and 5 Gy, although recent studies report that chromosomal abnormalities such as Dics can be detected following chronic or low-dose radiation exposure [10–13]. *In vitro* experiments using human peripheral blood (PB) lymphocytes revealed that the irradiation dose and chromosome aberration frequency correlate down to 20 mGy [14]. However, there is no useful method specifically designed for detecting chromosomal abnormalities following exposure to doses of 100 mGy or less, and the accuracy of estimation methods using the dose–response curves (DRCs) following exposure to the low doses remains unclear [15–18]. Furthermore, a DRC based on a sample from a single healthy individual is likely to be inadequate for estimating the exposure of a large number of people following a radiation exposure disaster. The DCA for biological dosimetry recommended by the International Atomic Energy Agency (IAEA) is affected by gender and age, as verified by the construction and comparison of DRCs by several laboratories [19, 20]. Chromosome translocation (Tr) analysis is strongly affected by factors such as age and smoking [21], again highlighting the problem of constructing a DRC based on a sample from a single healthy individual. In this study, we attempted to construct a DRC compatible with estimating a lower dose range than that which DRCs use conventionally. We therefore irradiated samples from five healthy individuals with eight gamma-ray irradiations doses from 0 to 1000 mSv. Here we present the three types of standard DRCs compatible with three methods. The first is a classical method for DCA, Giemsa staining. The second is the centromere-fluorescence *in situ* hybridization (centromere-FISH) method, which likely provides higher accuracy than Giemsa staining. The third is a painting method for chromosome translocation (Tr) analysis using three probes (one each for chromosome pairs 1, 2 and 4), which can estimate a radiation exposure dose received several years earlier. We also comment on the difficulty of assessing low radiation doses.

## MATERIALS AND METHODS

### Ethics statement

The samples and the medical records used in this study were approved by the Ethics Committee of the Fukushima Medical University School of Medicine (approval number 1577). Written informed consent was obtained from all participants for analysis of PB samples, and the methods were carried out in accordance with the approved guidelines of the Council for International Organizations of Medical Science [22].

### Blood samples

PB was collected from five healthy individuals: four males (23, 35, 44 and 55 years old) and one female (33 years old). None of the individuals had a history of smoking and had never been subjected to radiotherapy or chemotherapy for any disease.

### Gamma-ray irradiation and lymphocyte culture

PB samples were irradiated with gamma-rays (Gamma cell 40, Best Theratronics, Ottawa, Ontario, Canada; installation date: March, 2009.) at eight doses (0, 10, 20, 50, 100, 200, 500 or 1000 mGy). Plastic microtubes containing whole blood were irradiated at a distance of 16 cm at room temperature with gamma-rays from a $^{60}$Co radiation source (1.11 TBq) at a dose rate of 26.26$t$ (time: min) + 6.42 mGy per min, where 26.26 is the dose rate and 6.42 is the dose to the sample entering and leaving the irradiation source. The doses were measured using an ionization chamber detector for gamma-rays.

Mononuclear blood cells were isolated from heparinized PB samples using BD Vacutainer CPT tubes (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. Cells were suspended in RPMI 1640 medium (Nacalai Tesque, Kyoto, Japan) containing 20% fetal bovine serum (Equitech Bio, Keilor East, Australia), 2% phytohaemagglutinin-HA15 (Remel, Lenexa, KS, USA) and 60 μg/ml of kanamycin solution (Life Technologies, Carlsbad, CA, USA) in a 6-well plate. Lymphocytes were cultured in a 5% humidified $CO_2$ incubator at 37°C for 48 h. Colcemid solution (Wako, Osaka, Japan) was added (final concentration: 0.015–0.02 μg/ml) 2 h before cell harvest, then chromosome preparations were made according to a standard cytogenetic procedure [23].

### Staining

Each slide was stained using three methods. Giemsa staining was achieved by immersing the slide in 5% Giemsa (Merck Millipore, Darmstadt, Germany) solution for 15 min, then washing with distilled water and air drying. Centromere-FISH staining was achieved using Poseidon probe (KRATECH, Amsterdam, the Netherlands) according to the manufacturer's protocol, with slight modifications described previously [24]. Chromosome painting was achieved using a Customized XCP-Mix probe (Mix-#1R-#2G-#4RG; MetaSystems, Altlussheim, Germany) according to the manufacturer's protocol, as in our previous study [25]. Briefly, nuclear DNA was denatured by incubating the slides on a hot plate at 75°C for 2 min, followed by incubation overnight at 37°C in a humidified chamber to allow for hybridization. The glass coverslips were removed and the slides were washed in 0.4 × SSC at 72°C for 2 min. After draining, the slides were then washed in 2 × SSC/0.05% Tween-20 at room temperature (RT) for 30 s. Subsequently, the slides were briefly rinsed in distilled water to avoid crystal formation and then air dried at RT. Finally, nuclei were counterstained with Vectashield Mounting Medium containing DAPI (Vector, Burlingame, USA), and the slides were covered with a glass coverslip and sealed with nail polish.

### Image capture and scoring

Giemsa images and FISH images were captured in AutoCapt mode using two sets of AXIO Imager Z2 microscopes (Carl Zeiss AG, Oberkochen, Germany) equipped with CCD cameras and Metafer 4 software (MetaSystems GmbH, Altlussheim, Germany). Chromosome analysis was performed according to the International Atomic Energy Agency (IAEA) manual [9, 23] by three trained, experienced observers who were not informed of the irradiation dose.

Chromosomal aberrations in the FISH images were confirmed using fluorescence imaging software (Isis FISH Imaging System, ver. 5.4; MetaSystems GmbH). More than 2000 metaphases were scored in each Giemsa and centromere-FISH slide [24]. All observable aberrations were classified as dicentrics or multicentrometrics (chromosomes with three or more centromeres). Other chromosome- or chromatid-type aberrations were also recorded, such as rings, acentrics (aces), breaks and gaps. Metaphases with fewer than 45 centromeres were omitted from analysis. For translocation analysis, more than 5000 metaphases were scored on each slide [25]. Based on a previous report [26], we included apparent one-way translocations in the two-way translocation counts. For complex chromosomal abnormalities, the number of translocations was determined based on the number of color junctions (NCJs) [27]. We also recorded other chromosomal aberrations, such as Dics and aces and calculated the frequency of translocations across the whole genome using formulae published by IAEA [9]. For scoring of translocations, the formula used to calculate the frequency of translocations across the whole genome ($F_G$) was based on the formula using three colors (chromosome 1: red; chromosome 2: green; chromosome 4: yellow) for the detection of translocations as follows [9]:

$$F_G = F_{P(1+2+4)}/2.05[f_1(1-f_1) + f_2(1-f_2) + f_4(1-f_4) - (f_1f_2 + f_1f_4 + f_2f_4)]$$

$F_G$: the full genome aberration frequency,
$F_P$: the translocation frequency detected by FISH,
$f_p$: the fraction of genome hybridized, taking into account the gender of the subjects (female: $f_p = 0.2234$, male: $f_p = 0.2271$).

The proportion of the genome occupied by chromosomes 1, 2 and 4 is ~23%. Therefore, $F_G$ is determined by the following formula:

$$F_G = F_P \times 2.567 \text{ (Female)}$$

$$F_G = F_P \times 2.533 \text{ (Male)}.$$

In order to unify the cell numbers in the analysis, we determined $F_G$ in per 2000 cells equivalents, according to the above respective formula for females or males, respectively.

**Table 1. Average of dicentric chromosome results from five donors (Giemsa staining)**

| Dose (mGy) | Number of cells analyzed | Number of Dics[a] | Frequency of observed Dics[a] | 95%CI[b] (Dic-frequency) |
|---|---|---|---|---|
| 0 | 2023.4 | 2.8 | 0.001 | −0.0005–0.003 |
| 10 | 2026.4 | 4.2 | 0.002 | 0.0005–0.004 |
| 20 | 2020.8 | 2.6 | 0.001 | 0.0002–0.002 |
| 50 | 2051.8 | 4 | 0.002 | 0.0007–0.003 |
| 100 | 2020.4 | 4 | 0.002 | 0.0006–0.003 |
| 200 | 2026 | 7.2 | 0.004 | 0.001–0.006 |
| 500 | 2010.2 | 27.6 | 0.013 | 0.008–0.019 |
| 1000 | 2066.8 | 78 | 0.038 | 0.029–0.045 |

[a]Dicentric chromosomes per cell analyzed.
[b]Confidence interval.

**Table 2. Average of dicentric chromosome results from five donors (centromere-FISH)**

| Dose (mGy) | Number of cells analyzed | Number of Dics[a] | Frequency of observed Dics[a] | 95% CI[b] (Dic frequency) |
|---|---|---|---|---|
| 0 | 2011.2 | 1.6 | 0.001 | −0.00005–0.002 |
| 10 | 2015.2 | 2.2 | 0.001 | 0.0005–0.002 |
| 20 | 2018.8 | 3.2 | 0.002 | 0.0004–0.003 |
| 50 | 2026.2 | 4.8 | 0.002 | 0.001–0.003 |
| 100 | 2021 | 5.6 | 0.003 | 0.001–0.004 |
| 200 | 2026.6 | 12 | 0.006 | 0.004–0.008 |
| 500 | 2023 | 37.6 | 0.019 | 0.013–0.024 |
| 1000 | 2057 | 108 | 0.053 | 0.048–0.057 |

[a]Dicentric chromosomes per cell analyzed.
[b]Confidence interval.

## RESULTS

### Construction of dose–response curves for DCA (Giemsa staining and centromere-FISH)

Approximately 80 000 metaphases were analyzed for DCA: ~2000 metaphases per dose, and ~16 000 metaphases per individual. The average values from these analyses are shown in Tables 1 and 2. Compared with Giemsa staining, the frequency of Dic formation as a whole was higher in DCAs using FISH, except for 0 mGy and 10 mGy, and was higher than at 20 mGy using Giemsa staining (Table 1). Analysis of samples from each individual showed large variation in the frequency of Dic formation at 0 mGy by both methods, and the increase in the number of Dics formed did not correlate with the irradiation dose up to 100 mGy. In contrast, the number of Dics formed rapidly increased at irradiation doses above 200 mGy (see Supplementary Tables 1 and 2).

Based on these results, DRCs for Giemsa staining and centromere-FISH were constructed using DoseEstimate ver. 4.1 software [28]. The results from Giemsa staining were fit using the formula: $Y = 0.0013\ (\pm 0.0005) + 0.0067\ (\pm 0.0071) \times D + 0.0313\ (\pm 0.0091) \times D^2$ ($Y$: yield of chromosome aberrations, $D$: dose (Gy)), and the correlation coefficient ($r$) was 0.9985 (Fig. 1). The results from centromere-FISH were fit using the formula: $Y = 0.0010\ (\pm 0.0004) + 0.0186\ (\pm 0.0081) \times D + 0.0329\ (\pm 0.0104) \times D^2$ ($Y$: yield of chromosome aberrations, $D$: dose (Gy)), and the correlation coefficient ($r$) was 0.9998 (Fig. 2). Therefore, we constructed two types of DRCs that strongly correlated with the irradiation dose and the number of Dics

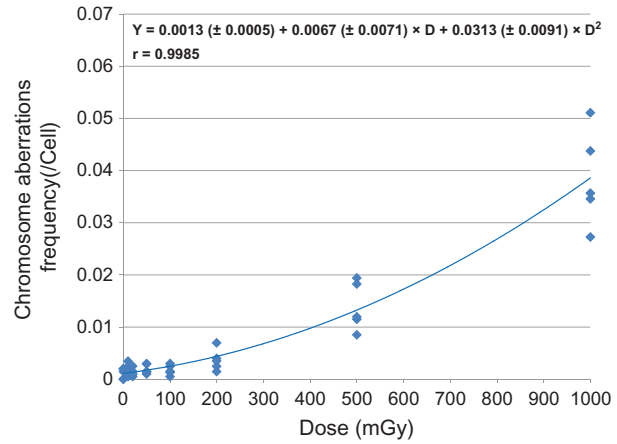


**Fig. 1. Dose–response curve for DCA analyzed by Giemsa staining. The frequencies of chromosome aberrations per 2000 cells in PB from five individuals induced by gamma-ray irradiation were plotted. Regression analysis using DoseEstimate ver. 4.1 software was calculated from the average value of the five samples. [$Y = 0.0013\ (\pm 0.0005) + 0.0067\ (\pm 0.0071) \times D + 0.0313\ (\pm 0.0091) \times D^2$, $r = 0.9985$] ($Y$: yield of chromosome aberrations, $D$: dose (Gy), $r$ = correlation coefficient.)**

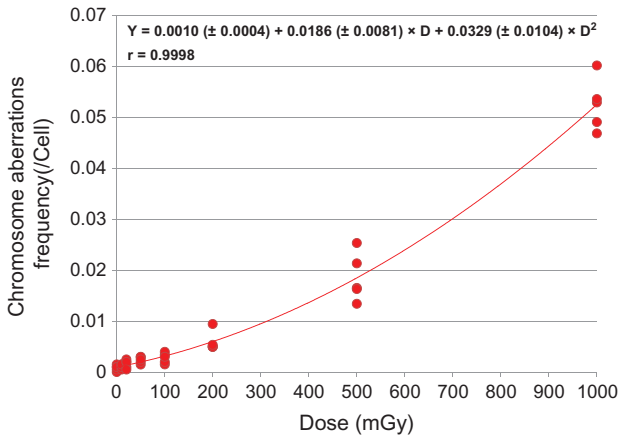


**Fig. 2. Dose–response curve for DCA analyzed by centromere-FISH. The frequencies of chromosome aberrations per 2000 cells in PB from five individuals induced by gamma-ray irradiation were plotted. Regression analysis using DoseEstimate ver. 4.1 software was calculated from the average value of the five samples. [$Y = 0.0010\ (\pm 0.0004) + 0.0186\ (\pm 0.0081) \times D + 0.0329\ (\pm 0.0104) \times D^2$, $r = 0.9998$] ($Y$: yield of chromosome aberrations, $D$: dose (Gy), $r$ = correlation coefficient.)**

formed using two methods. However, comparison of the DRCs between individuals indicated different slopes for each individual (see Supplementary Figs 1 and 2).

### Construction of a dose–response curve for chromosome translocation analysis

Approximately 200 000 metaphases were subjected to Tr analysis: ~5000 metaphases per dose, and ~40 000 metaphases per individual. The average values from these analyses are shown in Table 3. The average frequency of Tr at 10 mGy was higher than that at 20 mGy. Analysis of samples from each individual showed variation in the frequency of Tr. Variation was also observed in DCA, and the increase in the frequency of Tr did not correspond to the irradiation dose up to 50 mGy (see Supplementary Table 3).

Based on these results, the DRC for Tr analysis was constructed using DoseEstimate ver. 4.1 software [28]. The results were fit using the formula: $Y = 0.0053\ (\pm 0.0009) + 0.0259\ (\pm 0.0127) \times D + 0.0826\ (\pm 0.0161) \times D^2$ ($Y$: yield of chromosome aberrations, $D$: dose (Gy)), and the correlation coefficient ($r$) was 0.9995 (Fig. 3a). The slopes of DRCs of the five individuals showed no difference due to age or gender (see Supplementary Fig. 3), and therefore we succeeded in constructing a DRC that strongly correlates with the irradiation dose and the frequency of Tr. However, the frequencies of Tr at 0 mGy were higher than those of DCA (see Supplementary Figs 1 and 3), and therefore the DRC did not start from zero (Fig. 3a). Since the translocation frequency increases with age, we subtracted the frequency of age-dependent background reported by Sigurdson *et al.* [21] from the frequencies of Tr, thereby resulting in the DRCs starting essentially at zero (Fig. 3b). The DRCs obtained for Tr analysis focusing on the low-dose range are shown in Supplementary Fig. 3c and d.

## DISCUSSION

DCA is the most accurate biological dosimetry method for estimating radiation dose in humans and is used in conjunction with physical dosimetry, such as a personal dosimeter, during radiation exposure accidents. Radiation exposure estimation from the number of CAs, such as Dic and Tr, require the prior construction of a DRC using the frequency of CAs formed in lymphocytes at a series of irradiation doses. Since there are some differences in sample preparation protocols for CA analysis, it is desirable for each laboratory to have its own DRC. We constructed three types of DRC: one each for Giemsa staining and centromere-FISH for DCA, and one for a painting method for Tr analysis. Each curve was constructed using lymphocytes from five healthy individuals following irradiation with eight doses of gamma-rays. The curves showed good radiation dose responsiveness. Although there were large individual differences in the frequency of CAs for radiation doses of 100 mGy or less, dose estimation may be possible from the lower limit value of the 95% confidence interval (CI).

On the other hand, CAs were observed even in non-irradiated samples in both the DCA and Tr analyses, and there was no correlation between the frequency of CAs and the radiation dose up to 100 mGy. Therefore, variation was observed in the frequency of CAs, such as in the higher number of CAs formed during a low radiation dose compared with during a high radiation dose. These variances in the low-dose range of 100 mGy or less are likely due to individual confounding factors such as age. It is important to analyze a large number of cells to reduce the influence of confounding factors, and thus we analyzed 2000 metaphases in this study. A

**Table 3. Average of chromosome translocation analysis of five donors**

| Dose (mGy) | Number of cells analyzed | | Number of Trs | Frequency of observed Trs[b] | 95%CI[c] (Tr-frequency) |
|---|---|---|---|---|---|
| | Cell count of analysis | Cell equivalent[a] | | | |
| 0 | 5551.6 | 2176.2 | 9.6 | 0.004 | 0.001–0.008 |
| 10 | 5652.6 | 2215.8 | 13.4 | 0.006 | 0.004–0.008 |
| 20 | 5564.4 | 2181.2 | 13.0 | 0.006 | 0.002–0.01 |
| 50 | 5436.4 | 2131.1 | 14.6 | 0.007 | 0.003–0.011 |
| 100 | 5424 | 2126.2 | 19.2 | 0.009 | 0.004–0.014 |
| 200 | 6058.4 | 2374.9 | 34.2 | 0.014 | 0.011–0.019 |
| 500 | 5701.6 | 2235.0 | 81.4 | 0.036 | 0.033–0.04 |
| 1000 | 5197.4 | 2037.4 | 235.8 | 0.116 | 0.109–0.122 |

[a]Equivalent to full genome cell count (The formula is provided in the Materials and Methods.).
[b]Chromosome translocations per 100 cell equivalents.
[c]Confidence interval.

previous study reported a correlation between the radiation dose and the frequency of CAs, even at a low-dose of 20 mGy, based on the analysis of more than 5000 cells [14]. We therefore thought it advisable to analyze more than double that number of cells in this study. We did not believe that age adjustment was needed for DCA because chromosomes containing Dics are instable; thus lymphocytes containing Dics will not survive for years. However, a correlation between the number of Dics formed and the cumulative exposure dose was recognized in an analysis of residents from a high background radiation area in China [29]. In contrast, Tr is stable; thus lymphocytes containing Trs are expected to survive beyond the normal human life expectancy [30], which may be a confounding factor. The number of Trs increases with age and is affected by lifestyle factors such as smoking [21]. Furthermore, race, geographic region, and individual lifestyle are thought to affect the Trs formed [9]. We suggest that those several factors induced large variation between individuals in the frequency of Trs formed. Therefore, biological dosimetry using Tr analysis should be performed using age adjustment to prevent an erroneously high estimation of the number of CAs [21, 31]. In this study, we conducted age adjustment using the frequency of age-dependent background reported by Sigurdson *et al.* [21]. However, these frequencies were obtained from summarized analytical reports from several laboratories around the world and were not analyzed using the same method. Most analyses were conducted using a one-color probe, which is different from our three-color painting method. Therefore, it is necessary to obtain the background frequency for the Tr analysis based on the method of analysis. Furthermore, Shin *et al.* reported a difference in radiosensitivity between healthy individuals [32], suggesting it might be necessary to adjust the dose estimation by factors other than age.

The frequency of Tr formation in this study was higher than that of Dics in every sample. Theoretically, these two CAs should occur with the same frequency [33]. However, there is a difference in the frequency of occurrence [1], with the occurrence of Trs reported to be slightly higher than that of Dics [34], which we speculate is attributable to the different detection methods used for DCAs and Trs. All 46 chromosomes present in a cell are analyzed in DCA, whereas only six chromosomes (pair numbers 1, 2 and 4) are examined in Tr analysis, and the frequency of Trs formed is calculated based on the proportion of DNA in these chromosomes to the total DNA in the cell. Therefore, the former is an actual measurement and the latter is derived from a calculation formula.

In both DCA and Tr analysis, chromosomes are analyzed by stopping the cell cycle at the mitotic phase using colcemide. Chromosome fragments without a centromere formed in cells with Dics are not distributed to the poles during cell division, and thus cells with Dics cannot proceed beyond the mitotic phase. The G2/M checkpoint is believed to delay or pause the cell cycle; subsequently, the transition to the mitotic phase may be restricted [35]. Actually, when Dics and incomplete chromosome fragments lacking a telomere were formed in cells by irradiation, those cells were blocked at the G2/M checkpoint [36]. On the other hand, cells with Trs face no obstacle that affects cell division and so can transfer to the mitotic phase. Therefore, the number of Dics formed may be lower than the actual number of Dics formed when the cell cycle is stopped at the mitotic phase using colcemid; this may lead to a lower number of Dics compared with the number of Trs formed.

A comparison of the two DCA methods shows that the number of Dics observed by centromere-FISH tends to be higher than that observed by Giemsa staining, likely because the probe used to visualize the centromere region facilitates the discrimination of Dics, compared with Giemsa staining.

Recently, Suto *et al.* constructed a DRC based on Tr analysis from a single healthy individual [37]. In the present study, we constructed a DRC based on analyses of PB from five healthy individuals and thus recognized variation in the number of Trs formed, even for the same radiation dose. We propose that a DRC should ideally be constructed using samples from multiple healthy individuals of each age in intervals of 5 or 10 years. Sample preparation methods for radiation dose estimation using the number of CAs differ slightly between

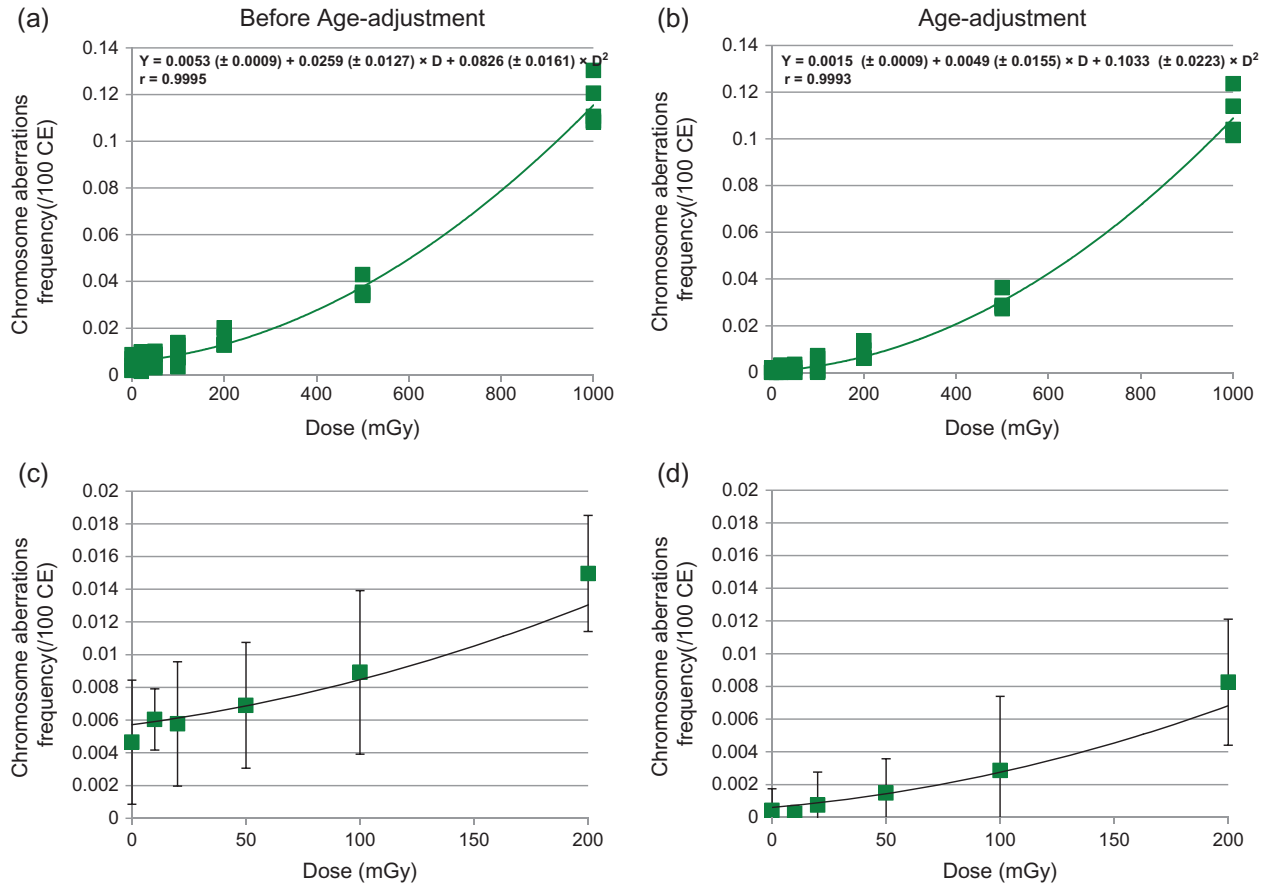


**Fig. 3. Dose–response curves for chromosome translocation analysis. The frequencies of chromosome aberrations per 2000 cells equivalents (Ces) in PB from five individuals induced by gamma-ray irradiations were plotted. (a) The dose–response curves before age-adjustment. Regression analysis using DoseEstimate ver. 4.1 software was calculated from the average value of the five samples. [$Y = 0.0053\ (\pm 0.0009) + 0.0259\ (\pm 0.0127) \times D + 0.0826\ (\pm 0.0161) \times D^2$, $r = 0.9995$] ($Y$: yield of chromosome aberrations, $D$: dose (Gy), $r$ = correlation coefficient). (b) The dose–response curves following age-adjustment. The regression analysis was [$Y = 0.0015\ (\pm 0.0009) + 0.0049\ (\pm 0.0155) \times D + 0.1033\ (\pm 0.0223) \times D^2$, $r = 0.9993$]. (c) The dose–response curve before age-adjustment focusing on the low-dose range. (d) The dose–response curve following age-adjustment focusing on the low-dose range.**

laboratories, and the criteria for determining CAs may be different; consequently, it appears that radiation dose estimation can differ between analyses of the same sample [19, 20, 38, 39]. Therefore, it will be necessary to construct globally unified sample preparation methods and criteria if we are to improve the accuracy of biological dosimetry.

We conclude that both DCA and Tr analysis following gamma-ray irradiation doses of 100 mGy or less, and especially of 50 mGy or less, may lack accuracy due to poor dose responsiveness in the number of CAs formed, as assessed by DCA and Tr analysis, and variation in the dose responsiveness of samples following these irradiation doses. On the other hand, as mentioned above, the accuracy would likely increase if we analyzed 5000 or more cells in the low-dose range. It would be unrealistic to expend such effort on biological dosimetry during an actual radiation disaster. It is therefore important that we learn how to estimate exposure doses of 100 mGy and higher quickly and accurately.

In conclusion, our study performing three different biological dosimetry assays simultaneously on PB from five donors highlighted the difficulty of biological dosimetry in the low-dose range.

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## SUPPLEMENTARY DATA

Supplementary data are available at the *Journal of Radiation Research* online.

## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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