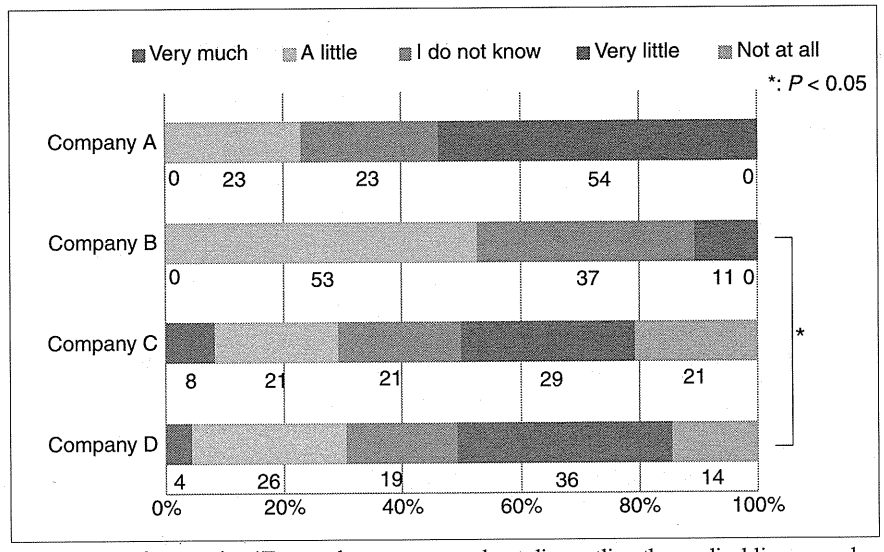

Fig. 13. The results of each company for question "Do you have concerns about dismantling the medical linear accelerator?"

at company C thought about information for determining the decay period than at company D ($P < 0.05$) (Fig. 12), and fewer maintenance workers at company D had concerns regarding dismantling medical linear accelerators than at company B ($P < 0.05$) (Fig. 13).

These differences among manufacturers indicate that the amount and quality of information available to maintenance workers are reflected by the educational activities conducted by the manufacturer. It has been confirmed that RC activities should be considered to compensate for differences between manufacturers, and it is also important to reduce gaps in risk perception among those involved.

Was RC successful?

The results of the present RC indicate that many maintenance workers enhanced their understanding of the decay period and effects of induced radioactivity on the human body and were satisfied with the information acquired. The present RC study was thus considered a success.

Okoshi et al. (2007) mentioned that one of the reasons that RC activities are not performed in the nuclear field, although the need and usefulness have been recognized, is that the RC methodology is not established practice; moreover, there is almost no experience with RC. How can we find a good solution to this situation?

The United States Environmental Protection Agency (USEPA) indicates the following principles as basic rules for advancing the appropriate RC (USEPA 1988):

- Rule 1. Accept and involve the public as a legitimate partner;
- Rule 2. Listen to the audience;
- Rule 3. Be honest, frank, and open;
- Rule 4. Coordinate and collaborate with other credible sources;
- Rule 5. Meet the needs of the media;
- Rule 6. Speak clearly and with compassion; and
- Rule 7. Plan carefully and evaluate performance.

Now consider whether the RC activities in the present study were appropriate in regard to rules 1–4 and 7. In terms of listening (and sharing information), the holding of meetings, the exchange of opinions and creation of a booklet were appropriate under rule 2. The representatives of the radiation therapy equipment manufacturers were members of the WG responsible for writing the society standards. These activities were appropriate in terms of information sharing and building a relationship of trust as partners between radiation therapy equipment manufacturers and user representatives under rules 1–4. The opinions of maintenance workers after the first RC were also investigated, and the authors evaluated the progress of how satisfied maintenance workers were with the information and the degree of information sharing. Then, using Plan-Do-Check-Act (PDCA), the second RC action plan was improved in consideration of previous results and was appropriate under rule 7. In other words, co-creation of the society standards and organization of the WG strengthened the foundations of RC activities.

Neutrality of the organizer performing the RC activities and of the organizations that cooperate with stakeholders is also required. Some of the representatives of the radiation therapy equipment manufacturers felt that there is a difference in the strength of the position between the manufacturer's and user's representatives. Therefore, it was necessary for the leader of the WG to determine rules without bias. To ensure neutrality, the chairperson of the WG was a medical staff member of a hospital that was not directly involved with use of the medical accelerator.

RC is required not only to enhance the good relationships among stakeholders but also to improve the RC

activities by using the PDCA cycle based on neutrality, following the basic rules of the USEPA, and to share good quality information.

Limitations

The present study is a practical RC, and it was not possible to have a control group. Therefore, there is a possibility that factors other than the intervention affect the results. Furthermore, the questionnaire was not validated.

CONCLUSION

Many maintenance workers enhanced their understanding of the decay period and effects of induced radioactivity on the human body and were satisfied with the information acquired. The present RC study was thus considered a success. The RC activities in the present study were appropriate with regard to USEPA principles as basic rules for advancing the appropriate RC. In particular, incorporating stakeholders in the organization of RC activities strengthened the foundations of the RC activities. It is important to improve the RC activities by using the PDCA cycle based on neutrality, following the basic rules of the USEPA, and sharing good quality information.

Acknowledgments—The present study was one of the WG activities and was mainly conducted by the Japanese Society of Radiological Technologists subcommittee on regulation issues.

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Short communication

Construction of a cytogenetic dose–response curve for low-dose range gamma-irradiation in human peripheral blood lymphocytes using three-color FISH

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ABSTRACT

In order to estimate biological doses after low-dose ionizing radiation exposure, fluorescence in situ hybridization (FISH) using three differentially colored chromosome painting probes was employed to detect exchange-type chromosome aberrations. A reference dose response curve was constructed using blood samples from a female donor whose lymphocytes consistently exhibited a low frequency of cells at the second mitosis under routine culture conditions. Aberration yields were studied for a total of about 155 thousand metaphases obtained from seven dose-points of gamma irradiations (0, 50, 100, 150, 200, 250 and 300 mGy). In situ hybridization was performed using commercially available painting probes for chromosomes 1, 2 and 4. With the aid of an automated image-capturing method, exchange-type aberrations involving painted chromosomes were detected with considerable accuracy and speed. The results on the exchange-type aberrations (dicentrics plus translocations) at the seven dose-points showed a good fit to the linear-quadratic model ($y = 0.0023 + 0.0015x + 0.0819x^2$, $P = 0.83$). A blind test proved the reproducibility of the reference dose–response relationship. In the control experiments using blood samples from another donor, the estimated doses calculated on the basis of the present reference curve were proved to be in good agreement with the actual physical doses applied. The present dose–response curve may serve as a means to assess the individual differences in cytogenetical radio-sensitivities.

1. Introduction

The dicentric chromosome assay (DCA) is currently regarded as one of the most validated techniques for evaluating the biological dose of ionizing radiation. The methodological standard of the assay has been accepted internationally [1,2]. In the DCA, the scoring of aberrant chromosomes in conventionally Giemsa-stained slides is, however, a time-consuming and labor-intensive method, especially in cases of exposure to low-dose ionizing radiation. Considering the characteristics of low frequency of the occurrence of induced dicentrics, one can predict the limitation of the conventional DCA as a biological dosimeter for low-dose irradiations. Therefore, the achievement of efficient scoring of aberrant chromosomes in a sufficient number of cells is a key technical procedure for obtaining statistically reliable estimates [3,4]. In recent years, automated chromosomal aberration detection systems have been developed in biodosimetry [5,6]. These high-throughput systems may provide us not only with accurate dose estimates, but also with the possibility of biodosimetric activities in cases of mass-casualty incidents to deal with the large number of samples in a limited time. Furthermore, newly devised molecular cytogenetic tools, namely, fluorescence in situ hybridization (FISH) technologies using probes for chromosomal centromere and telomere sequences (CT-assay) in combination with automated detection systems have been established [7].

In the present study, for the purpose of evaluating the biological effects of low-dose range ionizing radiation on human peripheral blood lymphocytes, the FISH method using three differentially colored chromosome-specific painting probes (3-color FISH; chromosomes 1, 2 and 4) was employed. The use of chromosome painting techniques as a means of biodosimetry has been the topic of considerable debate [8–33]. In the knowledge that it has limitations as well as advantages in detecting exchange-type chromosome aberrations as the endpoint of DNA double-strand break/repair processes [8,12,18,28,34–39], the present study revis-

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Table 1
Frequencies of exchange-type chromosome aberrations induced by low-dose range gamma-irradiations based on the results of the 3-color FISH examinations of a total of approximately 155 thousand metaphases (reference sample, Donor 1).

Dose (mGy)	Metaphases	Frequency ($\times 10^{-3}$)			Distribution (No. of TL + Dic per cell)				V/m[c]	iTL/iDic[d]
		TL[a]	Dic[b]	TL + Dic	0	1	2	3		
0	23826	2.015	0.378	2.392	23775	45	6	0	1.21	
50	36298	1.984	0.496	2.479	36214	78	6	0	1.13	−0.26
100	24431	2.169	0.778	2.947	24363	64	4	0	1.11	0.39
150	22966	3.266	1.524	4.790	22863	96	7	0	1.12	1.09
200	14457	3.804	2.075	5.880	14374	81	2	0	1.04	1.05
250	24763	4.886	2.988	7.875	24574	183	6	0	1.05	1.10
300	8116	5.175	4.189	9.364	8040	76	0	0	0.99	0.83

[a] TL: event of translocation. A sequential translocation among three chromosomes was scored as 2 translocations; a sequential event of dicentric and translocation was scored as 1 translocation and 1 dicentric.
[b] Dic: dicentric chromosome. A tricentric was scored as 2 dicentrics.
[c] V/m: variance to mean ratio (dispersion factor) for exchange-type chromosome aberrations (translocations plus dicentrics).
[d] iTL/iDic: ratio of induced translocation frequency and induced dicentric frequency. The induced aberration frequency at each dose-point was calculated by subtracting the background frequency (frequency at 0-Gy irradiation point).

ited the application of the FISH-painting technology and assessed the utility of the method from a practical viewpoint.

While in the CT-assay, the number of DNA double-strand breaks (DSBs) induced in lymphocytes is evaluated by the pattern of centromere and telomere signals, the 3-color FISH detects chromosomal exchanges by determining color junctions on chromosomal structures. This method provides us data on unstable-type aberrations (dicentrics) as well as stable-type aberrations (translocations). In practice, these exchange-type aberrations are detected efficiently with the aid of automated image capturing procedures. As has already been pointed out, the method has limitations in detecting complicated aberrations [8,20,28,36,39,40–43]. Even seemingly simple exchanges have the possibility of resulting from multiple complicated exchanges, leading to underestimation of aberration frequencies [18]. However, the yield of such complicated aberrations is presumed to be low in the low-dose-range irradiations [38]. The present study placed an emphasis on the efficacy of the 3-color FISH method as a biodosimetric tool, and aimed at opening up further possibilities for evaluating cytogenetic effects of low-dose range irradiations on human lymphocytes by examining a large number of metaphases.

The authors first constructed a dose response curve on the basis of the results of exchange-type aberration yields at seven dose points (0, 50, 100, 150, 200, 250 and 300 mGy) obtained from a total of 155 thousand metaphases. By blind testing for two different dose-points, the reproducibility of the dose estimation based on the present dose–response curve was assessed. Then, a preliminary investigation was made to assess the validation of the present reference dose response curve by comparing with data obtained from blood samples from another donor.

2. Materials and methods

2.1. Donor selection

For the construction of the reference dose response curve, the following criteria were employed to choose a blood sample donor: (1) who had never been subjected to any of the known clastogenic overexposures, (2) whose blood lymphocytes exhibited a low incidence of the second metaphase (MII) cells under routine culture conditions (48-h culture with a final 2.5-h colcemid treatment), and (3) who had no habit of smoking. Following the criteria, blood samples were collected from a healthy female (47 year of age) after obtaining her informed consent. The frequency of MII cells 48 h after culture initiation was already known from repeated culture experiments to consistently show below 3%, ranging mostly from 1.0 to 2.5%.

2.2. Gamma irradiation and lymphocyte culture

Plastic tubes containing 3-ml whole blood were vertically irradiated at the 101-cm distance on a water phantom (15-cm depth) at room temperature with gamma-rays from a ^{60}Co radiation source at a dose rate of 5 mGy per second. The doses were measured using an ionization chamber detector for gamma-rays (Exposure Ratemeter, AE-132a; Ionization Chamber, C-110, Applied Engineering Inc., Tokyo, Japan), which was calibrated accurately. The uncertainty of the calibration was 1.4%, which is traceable to the national standards of Japan (National Institute of Advanced Industrial Science and Technology, Tokyo, Japan). Irradiations with doses higher than 300 mGy (500, 750 and 1000 mGy) were also conducted to examine the applicability of the dose–response curve established on the basis of the dose range of 0–300 mGy irradiations for the dose estimation of such higher doses.

From each blood sample, a mononuclear cell fraction, containing more than 90% lymphocytes, was separated by the gradient separation method using BD Vacutainer CPT tubes (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. The cells (approximately 5×10^6) for each dose-point were cultured in a culture tube containing 6 ml medium composed of RPMI1640 containing HEPES buffer (Life Technologies, Carlsbad, CA, USA), supplemented with 20% fetal bovine serum (SAFC Biosciences, Lenexa, KS, USA), 3% phytohemagglutinin (Remel, Dartford, Kent, UK) and 100 μg/ml kanamycin (Life Technologies). The cultures were kept in a CO_2 incubator at 37 °C for 45.5 h. Then, colcemid solution (Wako, Osaka, Japan) was added (final concentration: 300 ng/ml) and continued to culture for additional 2.5 h. The cells were treated with a hypotonic solution (0.075 M KCl) for 25 min, and fixed with acetic acid/methanol (1:3) fixative three times. In total, seven sets of experiments with differing combinations of dose-points were conducted. In each experiment, the frequency of MII cells was examined for an additional non-irradiated sample by treating with 5-bromo-2′-deoxyuridine (BrdU, 30 μM) (Sigma–Aldrich, St. Louis, MO, USA) from the initiation of culture.

Chromosome preparations were made by the standard method. The concentration of cells in the acetic acid/methanol fixative was adjusted to generate 500–1000 well-spread metaphases per slide glass. Commercially available fluorophore-labeled probes (chromosome 1, Texas Red; chromosome 2, FITC; chromosome 4, Texas Red plus FITC) (MetaSystems GmbH, Altlussheim, Germany) were used. In situ hybridization of the painting probes was performed as described by the manufacturer's protocol with slight modifications. In brief, after the pretreatment in 2× SSC solution (for 30 min at 70 °C and for 20 min at room temperature), the slides were rinsed in a 70% ethanol solution. Then chromosomes were denatured in an alkaline solution (0.1 M sodium hydroxide 70%; 30% ethanol) for 1 min, and dehydrated in an alcohol series (70, 95, and 99.5% ethanol, 1–3 min each). From the same donor, blood samples were collected for blind testing. Two dose-point experiments (127 mGy and 225 mGy) were conducted.

2.3. Aberration detection and scoring

Fluorescence metaphase images were captured in the AutoCapt mode by using two sets of cytogenetic image scanning system (Axio Imager Z2, Carl Zeiss Microscopy, Oberkochen, Germany; CoolCube 1, Metafer ver. 4, MetaSystems GmbH, Altlussheim, Germany) equipped with appropriate filter sets.

First, images of metaphases containing three pairs of differentially colored chromosome homologs (chromosome 1, red; chromosome 2, green; chromosome 4, yellow) were examined in terms of structural chromosome aberrations. The total chromosome count of each metaphase spread was not examined, except for cases when karyotype analyses were necessary for examining complicated structural chromosome aberrations [8]. Then, aberration types were determined by merging these painted chromosome images with DAPI (4′,6-diaminido-2-phenylindole)-stained ones. Under the present experimental conditions, centromeric heterochromatins were distinctively stained with DAPI, enabling discrimination between translocated chromosomes (single centromeres) and dicentrics (two centromeres). The exchange-type aberrations were tentatively divided into the following three categories: (1) simple aberrations including both complete chromosome exchanges (reciprocal translocations and dicentrics associated with fragments) and seemingly incomplete exchanges (non-reciprocal translocations and dicentrics with no associated fragments), (2) complex aberrations including sequential exchanges between multiple chromosomes, and (3) insertions,

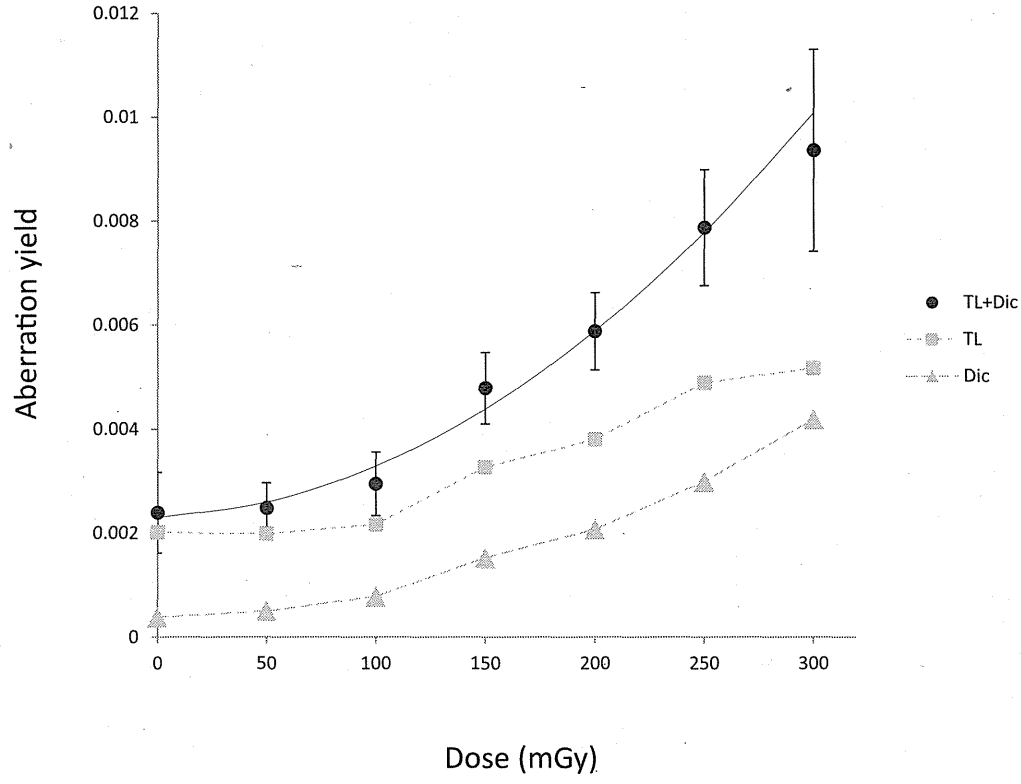

Fig. 1. Reference dose–response relationship of exchange-type chromosome aberrations (dicentrics plus translocations) examined by 3-color FISH in gamma-irradiated cultured peripheral blood lymphocytes from Donor 1. $Y = 0.0023\ (\pm 0.0003) + 0.0015\ (\pm 0.0058) \times D + 0.0819\ (\pm 0.0225) \times D^2$, $P = 0.83$. *Y*: yield of chromosome aberrations, *D*: dose (Gy). Frequencies of dicentrics plus translocations (●), dicentrics (▲) and translocations (■) are shown. Bars indicate 95% confidence intervals.

rings and highly complicated aberrations exemplified by so-called rogue cells [44]. Sequential chromosome exchanges in the category (2) involving three chromosomes [for instance, t(A;B;C)(q;q;q) in which the segments on the long arms of chromosomes A, B, and C have been translocated onto the long arms of chromosomes B, C, and A, respectively] were scored as double exchange-type aberrations. To establish a dose response curve, aberrations categorized as (3) were not included in the present analyses [28]. By using MetaClient ver. 1.1.1 (MetaSystems GmbH), metaphases were displayed on two large computer monitors, each of which showed eight frames of images (Supplementary Fig. 1). A pair of investigators examined the metaphases; when aberrant chromosomes were found, the two investigators crosschecked the aberrations in question on enlarged images using a fluorescence imaging software, Isis FISH Imaging System, ver. 5.4 (MetaSystems GmbH).

The chromosome slides prepared for the blind testing were coded and the metaphase images were examined by a scoring investigator, to whom no information was given about the doses, the number of dose points and even the mode of exposure (for instance, the mixed samples simulating partial body irradiations). In total, more than 5000 metaphases were scored in each of two-dose experiments. For assessing the reproducibility of the dose estimation experiments, exactly the first 5000 cells were used.

In order to assess the validation of the present dose response curve, a set of experiments was performed using blood samples from another donor, a 40-year old healthy female. In this case, isolated lymphocytes were cultured in the presence of BrdU (30 μM) for examining cell proliferation kinetics. As for the detection of MII cells, chromosomal slides were aged for 3–5 days. A modified procedure of the fluorescence-plus-Giemsa (FPG) method [45] was applied to reveal chromatid differentiation stain patterns.

3. Results

3.1. A reference dose response curve

Table 1 and Fig. 1 show the frequencies of exchange-type chromosome aberrations induced by low-dose range gamma-irradiations based on the results of the examinations of a total of approximately 155 thousand metaphases. Frequencies of all types of chromosome aberrations and examples of metaphase images are demonstrated in Supplementary Table 1 and Supplementary Figs. 1 and 2. The regression analyses by the DoseEstimate ver. 4.1 software [46] demonstrated that the datasets for dicentrics $[Y = 0.0004\ (\pm 0.0001) + 0.0008\ (\pm 0.0028) \times D + 0.0398\ (\pm 0.0117) \times D^2]$, translocations $[Y = 0.0019\ (\pm 0.0003) + 0.0011\ (\pm 0.0050) \times D + 0.0407\ (\pm 0.0188) \times D^2]$, and dicentrics plus translocations $[Y = 0.0023\ (\pm 0.0003) + 0.0015\ (\pm 0.0058) \times D + 0.0819\ (\pm 0.0225) \times D^2]$ gave a good fit to the linear-quadratic model ($P = 0.99$, $P = 0.83$ and $P = 0.83$, respectively); *Y*: yield of chromosome aberrations, *D*: dose (Gy). The variance to mean ratios ranged from 0.99 to 1.21. Considering the low frequency of each exchange-type aberration, the combined frequency was regarded as a suitable indicator for the practical dose estimation using a limited number of cells. Consequently, in the further analyses, the combined frequency at each dose-point was used as the primary indicator for constructing the reference dose–response curve. As shown in Fig. 1, 95% confidence intervals (CIs) for the exchange-type aberration frequencies at 0-, 50- and 100-mGy dose-points overlapped each other. According to the analysis of aberration frequencies among these three dose-points, no significant differences were found ($P > 0.10$, the approximate binomial two-sample test). In contrast, differences between aberration frequencies for 150 mGy and those for 100 mGy or less were statistically significant ($0.01 > P$). In other words, the combined yields of exchange-type aberrations could reach the statistically significant level at 150 mGy irradiations when compared to the baseline frequency. This holds true for the frequencies of dicentrics and those of translocations. This was concordant with

Table 2
Dose estimation of the blind test based on frequencies of exchange-type chromosome aberrations (translocations and dicentrics) involving painted chromosomes induced by gamma-irradiations (Donor 1) for validation of the reference dose–response curve.

Test	Metaphases	Frequency ($\times 10^{-3}$)			Actual dose (mGy)	Estimated dose (mGy)	95% confidence interval (mGy)
		TL[a]	Dic[b]	TL + Dic			
test 1	5000	2.600	0.600	3.200	127	96 ± 52	0–198
test 2	5000	3.200	2.800	6.000	225	204 ± 43	120–288

[a] TL: event of translocation. A sequential translocation among three chromosomes was scored as 2 translocations; a sequential event of dicentric and translocation was scored as 1 translocation and 1 dicentric.
[b] Dic: dicentric chromosome. A tricentric was scored as 2 dicentrics.

the previously reported level of the minimum detectable acute dose of radiation on the basis of varied frequencies of chromosome aberrations in non-irradiated adult individuals [17].

3.2. Validation of the reference dose response curve

(1) A blind test using blood samples from the same donor (Donor 1) was performed to assess the reproducibility of the present reference dose–response equation (Table 2, Supplementary Table 2). Estimated doses calculated based on 5000 metaphases for each dose-point showed a good agreement with the physical doses. Estimated doses of Test 1 (actual dose, 127 mGy) and the Test 2 (actual dose, 225 mGy) were 96 ± 52 mGy (95% CI, 0–198 mGy) and 204 ± 43 mGy (95% CI, 120–288 mGy), respectively, validating the reproducibility and reliability of the present dose–response equation. (2) The applicability of the reference dose response curve was assessed for cytogenetic results obtained from blood samples from another individual (Donor 2) (Table 3, Supplementary Table 3). The cultures for every dose-point exhibited a consistently low frequency of MII cells (1.3–2.1%) (Supplementary Table 3). To compare the results with the reference data, aberration yields in the first metaphase cells were used. The yields of aberrations were higher than those of the reference at most of the dose-points due to the higher baseline frequency in Donor 2, but were in good agreement with those estimated from the reference curve (Table 3). (3) At higher dose-points (500-, 750- and 1000-mGy), complex-type aberrations involving more than three chromosomes drastically increased. Such complicated aberrations were not included in the frequencies of exchange-type aberrations (Supplementary Table 1) because of the difficulty in precisely evaluating chromosomal exchange numbers. Although the observed values were not significantly different from the expected ones (chi-square, $P > 0.8$), the values increasingly deviated from the extrapolated reference curve with the increase of radiation dose (Supplementary Fig. 3).

4. Discussion

To elucidate the relationship between biological effects and absorbed radiation at low doses in cultured human peripheral blood lymphocytes, a number of in vitro experiments using chromosome aberrations as biological markers have been documented [9,18,19,27,29,47–55]. In the DCA, the accuracy of aberration detection is largely dependent on the quality of preparations, as well as on the criteria of metaphase selection and aberration determination. According to the reports on interlaboratory comparisons, researchers noticed that the raw data on dicentric frequencies obtained from the same irradiation experiments were often discordant among participating laboratories in the surveys [22,56,57]. The discordance may partly be attributed to technical factors including differences in the scoring criteria of cells that were not rigidly identical among the laboratories involved. To solve these problems, a semi-automated system has been introduced by collaborative work in European Laboratories [5]. Furthermore, an automated dicentric scoring system has been developed as a high-throughput tool [6,58]. Currently, however, the applicability of the method has not yet been extensively assessed for the cytogenetic effects of low-dose range irradiations.

In the present study, the chromosomal dose response relation in the low-dose range ionizing radiation (0–300 mGy) was described on the basis of the examination of approximately 155 thousand metaphases. The 3-color FISH enabled unequivocal detection of exchange-type chromosome aberrations except for the cases of those between homologous chromosomes and those involving small-sized chromosomal materials that are below the microscopic resolution limit [10,59]. When compared with other biodosimetric tools, the 3-color FISH method has advantages as well as limitations. In the 3-color FISH method, exchanges involving colored chromosomes were detected with considerable speed and accuracy. Unlike the time-consuming examination of dicentric chromosomes in conventionally Giemsa-stained preparations, the exchange-type aberrations were detected easily and rapidly even by those who are not skilled in cytogenetical technologies. For instance, metaphases containing overlapping and/or twisting chromosomes did not cause difficulties in determining exchange-type chromosome aberrations. Furthermore, in most cases of metaphase scoring, the scorers could focus on watching the three painted chromosome pairs, and not on the total diploid chromosome set. On average, eight frames of metaphase images could be examined in less than 15 s including the time for data recording processes. Subsequently, two investigators could examine 16 frames of metaphase images in less than 15 s (namely, the scoring efficiency was expressed as one metaphase image per second). In addition, the time needed to capture chromosomal images was significantly reduced by the automated microscopic metaphase-finding procedure. This semi-automated detection strategy facilitated obtaining reliable data by efficiently increasing the number of cells examined. The development of sophisticated software adapted to the fully automated 3-color FISH method will make more efficient scoring possible.

In the 3-color FISH method, painted chromosomes were limited to the following three pairs: chromosomes 1, 2 and 4. This may lead to underestimating exchange-type aberrations because of the disability of detecting "hidden" exchanges generated by multi-step exchanges as described previously [42]. The multiplex fluorescence in situ hybridization (M-FISH) method using 24-color chromosome paints may reveal chromosomal exchanges in full [38,39]. However, the method is inefficient for scoring the chromosomal aberrations induced by low-dose range radiation in view of the time and cost needed for examining a sufficient number of metaphases. The DNA amount of the three painted chromosome pairs comprises about 23% of the total DNA of the human genome (Homo sapiens GRCh38.p3, available at http://www.ncbi.nlm.nih.gov/assembly/GCF_000001405.29). A question arises as to whether the data obtained from such three large chromosomes represent chromosomal aberration events that take place in the whole genome. It has been accepted that the involvement rate of painted chromosomes in total exchange events is proportional to

Table 3
Frequencies of exchange-type chromosome aberrations in 1st metaphases induced by low-dose range gamma-irradiations based on the results of the 3-color FISH examinations of a total of approximately 45 thousand metaphases (control sample, Donor 2).

Dose (mGy)	1st Metaphases	Frequency ($\times 10^{-3}$)			Distribution (No. of TL + Dic per cell)			V/m[c]	Estimated dose (mGy)	95% confidence interval (mGy)
		TL[a]	Dic[b]	TL+Dic	0	1	2			
0	7738	2.585	0.258	2.843	7716	22	0	1.00	73 ± 52	0–175
50	6220	2.894	0.643	3.537	6198	22	0	1.00	103 ± 45	14–192
100	6791	3.092	0.736	3.829	6767	22	2	1.15	128 ± 41	47–208
150	5819	3.265	1.203	4.468	5793	26	0	1.00	154 ± 41	73–235
200	5603	4.997	3.034	8.031	5559	43	1	1.04	256 ± 44	169–342
250	7278	7.145	3.435	10.580	7203	73	2	1.04	309 ± 46	218–400
300	5221	4.597	4.405	9.002	5177	41	3	1.12	277 ± 46	187–367

[a] TL: event of translocation. A sequential translocation among three chromosomes was scored as 2 translocations; a sequential event of dicentric and translocation was scored as 1 translocation and 1 dicentric.
[b] Dic: dicentric chromosome. A tricentric was scored as 2 dicentrics.
[c] V/m: variance to mean ratio (dispersion factor) for exchange-type chromosome aberrations (translocations plus dicentrics).

the DNA content of targeted chromosomes [16,39,60–62], although there were reports suggesting chromosomal differences in the radiation sensitivity [63–66]. According to the assumption proposed by Lucas et al. [67,68], the genome-equivalent chromosomal exchanges deduced from the results of the 3-color FISH are about 39% of the total exchanges in the genome [50].

For establishing the reference curve, the culture procedures did not include the treatment of BrdU. The application of BrdU enables the demonstration of cells in their second mitosis. Therefore, the BrdU treatment procedure for detecting MII cells has been included in the standard protocol of radiation cytogenetics [1,2]. BrdU-incorporated DNA strands are known to be sensitized to ionizing radiation. On the other hand, the addition of BrdU after G0-irradiation has been perceived not to influence the yield of chromosomal aberrations. Although no evidence could be given in the present study regarding the influence of the addition of BrdU on the induction/repair of DNA DSBs, such a potentially genotoxic factor was eliminated to highlight the chromosomal aberration induction by low-dose radiation effects in constructing the first reference dose response curve. Comparative analyses using blood samples from Donor 2 were performed on the assumption that the influence of BrdU on the yield of chromosomal aberrations was negligible. Although the conclusive description should be reserved due to this assumption, the frequencies of dicentrics, translocations and the combined frequencies obtained from the samples of Donor 2 gave estimates in good agreement with those estimated from the reference curve at every dose-point ($P > 0.8$).

Because of cytokinesis failure, cells bearing unstable chromosome aberrations such as dicentrics have a probability of being lost during mitosis. Inclusion of MII cells with declined unstable aberrations in a cell population may lead to underestimation of the yield of exchange-type chromosome aberrations. In an attempt to address this issue, a preliminary study by 3-color FISH was performed to examine the frequencies of exchange-type aberrations in MII cells. In order to analyze sufficient number of cells containing chromosome aberrations, 2-Gy gamma-irradiated lymphocytes from Donor 1 were cultured with the addition of BrdU for 48 h and 72 h to detect exchange-type aberrations in MI and MII cells, respectively. The results were as follows: (1) the yield of translocations in MII cells was not significantly different from that in MI cells, and (2) the yield of dicentrics in MII cells was considerably higher than 50% of the dicentric yield of MI cells. Accurate quantification of exchange-type aberrations induced by high dose irradiation, however, was difficult due to the pronounced increase of complex-type exchanges. Therefore, the results on the frequencies of exchange-type aberrations in MII cells observed in cells exposed to high-dose radiation have to be regarded tentative. A trial calculation was made on the basis of these tentative results. The resulting dose–response equations showed that the inclusion of exchange-type aberrations derived from MII cells (1–2% of total cells analyzed) did not make a significant difference from the present dose–response equations. Considering the individual differences in the proliferation kinetics of cultured peripheral blood lymphocytes, a practical protocol for the evaluation of radiation-induced chromosome aberrations is expected to be devised for further studies.

Besides the above-mentioned technical limitations, the 3-color FISH method includes problems concerning the expensive cost of painting probes. In the present study, commercially available painting probes were used. It has already been pointed out that the cost of such probes would be a practical obstacle in realistic biodosimetric scenes. To cope with this problem, we are currently optimizing the hybridization conditions of very low-cost DNA probes for the 3-color FISH that are in-house products. This will provide a suitable reagent for the practical application of the 3-color FISH.

According to the previously reported results of DCA using dicentrics plus rings as markers, aberrations increased linearly from 0 up to 50 mGy and the yields were significantly above the control frequency from the dose of 20 mGy [53]. This was in sharp contrast to the present 3-color FISH results, in which no significant differences between 0 and 50 mGy were detected. A major issue was left unsolved in the present study as to what extent individual differences in cytogenetic radio-sensitivities exist as measured by the induction of exchange-type aberrations. As shown in the results of Donor 2, the yields of exchange-type aberrations at every dose-point exhibited a tendency of higher values (Table 3 and Supplementary Table 3). Considering the higher baseline frequency of Donor 2 when compared with that of Donor 1, the yields of induced exchange-type aberrations were not significantly different between these two individuals. To solve this crucial issue regarding individual differences in radio-sensitivity, the present dose–response curve will serve as the reference for comparative investigations focusing on further systematic cytogenetic analyses based on a sufficient number of samples.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.mrgentox.2015.10.002.

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