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TECHNICAL ADVANCE

Anti-CENP-C Antibody-Based Immunofluorescence Dicentric Assay: Radiation Dose-Response, Validation Studies, and Radiation Dose-Dependency on Sister Centromere Fluorescence

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Dicentric chromosome assay (DCA) is the most accepted cytological technique for the purpose of biological dosimetry in radiological and nuclear accidents, however, it is not always easy to evaluate dicentric chromosomes because of the technical difficulty in identifying dicentric chromosomes on Giemsa-stained metaphase chromosome samples. Here, we applied an antibody recognizing centromere protein (CENP) C, CENP-C, whose antigenicity is resistant to the fixation with Carnoy's solution. Normal human diploid cells were irradiated with various doses of ^{137}Cs γ rays at 1 Gy/min, treated with hypotonic solution, fixed with Carnoy's fixative, and metaphase chromosome spreads were stained with anti-CENP-C antibody. Dose-dependent induction of dicentric chromosomes was confirmed between 1 and 10 Gy of γ rays, and the results were compatible with those obtained by the conventional Giemsa-stained chromosome samples. The CENP-C assay also uncovered the difference in the fluorescence from the sister centromeres on the same chromosome, which was more pronounced after radiation exposure. Although the underlying mechanism is still to be determined, the result suggests a novel effect of radiation on centromeres. The innovative protocol for CENP-C-based DCA, which enables ideal visualization of centromeres, is simple, effective and reliable. It does not require skilled examiners, so that it may be an alternative method, avoiding uneasiness of the current DCA using Giemsa-stained metaphase chromosome samples.

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INTRODUCTION

In radiological and nuclear accident, immediate dose estimation for the victims is critical for prompt and adequate medical treatments and also to predict the patients' prognosis, so that physical as well as biological dosimetry have been applied for the assessment of radiation dose. The gold standard for the current biological dosimetry is the dicentric chromosome assay (DCA), which uses metaphase chromosomes fixed with Carnoy's fixative and stained with Giemsa's solution (*1–4*). While the method is simple and inexpensive, specialized experience and training are required for identifying dicentric chromosomes. In recent years, the fluorescence in situ hybridization (FISH) method using fluorescence probes targeting centromeres and telomeres has been developed. Although difficulty in detecting dicentric chromosomes is significantly improved, complicated procedures, availability of expensive FISH probes, and disruption of chromosome structure by heat treatment limit general usage of the FISH-based DCA under the radiological and nuclear accident situations (*5–7*). Thus, the establishment of a simple, easy, and convenient standardized protocol for the DCA is still to be pursued. Considering that the current DCA evaluates dicentric chromosomes based upon chromosome morphology, it must be beneficial for the DCA to establish a way to visualize centromeres. In fact, it has been shown that centromere-FISH staining with centromere-specific FISH probes significantly improves detection of dicentric chromosomes (*8*). Therefore, it is highly desirable to establish a protocol enabling a direct observation of centromeres.

The centromere is an essential structure consisting of α-satellite DNA and proteins, on which the kinetochore is assembled. The kinetochore is a protein complex, to which the spindle fibers attach, organizes the correct chromosome segregation during metaphase. Proteins comprising the kinetochore include various types of proteins collectively called centromere proteins (CENPs). The first identification of the centromere proteins came from the analysis of the sera obtained from the patients with scleroderma. Subsequent study defined three CENPs, CENP-A, CENP-B, and CENP-C, all of which were constitutively localized at the centromere (*9*). It now becomes to be clear that CENP-A is a histone H3 variant and connects centromere-specific nucleosome to kinetochore proteins (*10–12*). Histone H3 is one of the four histones consisting of nucleosome core. Generally, two of each histone, H2A, H2B, H3, and H4, form nucleosome core octamer, but in the centromere regions, CENP-A replaces one or two of histone H3 and gives rises to unique nucleosome cores (*13*). Association between CENP-A and kinetochore proteins is critical for proper establishment of the functional centromere. It is well recognized that there are two kinetochore regions, an inner kinetochore and an outer kinetochore, in which the former contains many CENP proteins, termed the constitutive centromere associated network (CCAN), and the latter forms the KNL1 complex, Mis12 complex and Ndc80 complex (KMN) network that binds directly to spindle microtubules (*14–17*). CENP-A interacts with some of the CCAN components, and one of which is CENP-C. CENP-C is composed of 943 amino acids and has two domains including the central region that binds to DNA and the C-terminal region that is required for dimerization (*18*). Thus, CENPs, especially CENP-C, should be the candidate for the target protein visualizing functional centromeres.

Fluorometric analysis has been applied for cytogenetics for a long time as it enables sensitive visualization of concealed chromosome aberrations. For example, the fluorescence-tagged probes for centromeric and telomeric DNA is applicable to the chromosome samples fixed with Carnoy's fixative (Methanol: Acetic acid = 3:1), which is known as the FISH technique. However, the fixation with Carnoy's solution negates recognition of chromosomal proteins by the most antibodies, preventing the usage of immunofluorometric assay. Although the Cytospin-spreaded chromosome samples can be applicable, the Cytospin technology is not available everywhere. Thus, the protocol using fluorometric detection of proteins, which are resistant to Carnoy fixation, has long been anticipated.

Previously, a report described that CENP-C did not lose its antigenicity after the fixation with Carnoy's solution, indicating that it could be applicable to immunofluorescence staining (*19*). The study examined several antibodies recognizing CENPs and found that different antibodies against CENP-C gave positive signals in cells treated with Carnoy's fixative. While the study suggested potential usage of the CENP-C antibody in the DCA, no further examination was performed. Therefore, we verified the usefulness of the CENP-C antibody-based DCA by comparing its dose-response with that obtained by the conventional Giemsa-stained chromosome samples. Our results demonstrated that both assays provided comparable results. The CENP-C assay also showed that the difference in the fluorescence from the sister centromeres on the same chromosome became more pronounced after radiation exposure, suggesting that radiation exposure might have an impact on centromeric protein organization. Thus, the CENP-C antibody-based DCA is a practicable alteration for a method of dicentric chromosome analysis. It is also indicated that the CENP-C assay could be a new clue to study an effect of radiation on centromere function.

MATERIALS AND METHODS

Cell Culture and γ Irradiation

Normal human diploid fibroblasts (NHDFs), immortalized by introduction of the *hTERT* gene (BJ1-hTERT, CRL-4001, ATCC, Manassas, VA) (*20*), and MOLT-4 cell line (JCRB Cell Bank, Tokyo) (*21*) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Wako Pure Chemicals, Osaka) supplemented with 10% fetal bovine serum (FBS) (Thermo Trace, Tokyo) at 37°C. Cells were maintained exponentially by subculturing them every 2 to 3 days, and in most experiments, 1.0×10^6 cells were seeded into T75 flasks (75 cm^2). Cells

Cells cultured in a T75 flask were placed in a ^{137}Cs-equipped γ irradiator (Pony Industry Co., Ltd, Osaka), and they were irradiated with different doses of γ rays (0, 1, 2, 4, 6, and 10 Gy) at a dose rate of 1 Gy/min at room temperature. Dose rates were measured by the glass dosemeter element system (DoseAce FGD-1000, Chiyoda Technol Corp., Tokyo), and the radiation field uniformity was shown in Supplementary Fig. S1 (https://doi.org/10.1667/RADE- 22-00050.1.S1).

Chromosome Preparation

Immediately after irradiation, colcemid (Invitrogen, New York) was added to the culture medium at a final concentration of 0.05 μg/ml and incubated them in a 5% CO_2 incubator at 37°C for 24 h. Chromosome samples were prepared following standard cytogenetic procedure (*3*). Briefly, metaphase cells were harvested by tapping the flasks and collected by centrifugation at 1,500 rpm for 5 min at room temperature. The supernatant was removed and replaced by a hypotonic solution of 0.075M KCl. Cell pellets were resuspended by mild pipetting, and cell suspension was incubated for 25 min at on ice. Then, freshly prepared Carnoy's solution (methanol:acetic acid = 3:1) stored on ice was added. Cells were kept on ice for 30 min, and finally, cell suspension was spotted onto a slide glass. The slides were air-dried for immunofluorescence, or immersed in 4% Giemsa staining solution prepared in distilled water for 15 min at room temperature. Then, the samples were rinsed in distilled water, air-dried, and mounted on the slide glasses with EUKITT (O. Kindler GmbH, Gartringen, Germany).

Immunofluorescence

Immunofluorescence on mitotic cells was performed using NHDFs cultured on cover slips (22 × 22 mm, Matsunami Glass, Tokyo). Cells (5×10^4 cells/slip) plated onto the cover slips were incubated for 48 h before fixation with cold-methanol or with Carnoy's solution. For immunofluorescence of chromosome spreads, metaphase chromosome samples stored at room temperature were rehydrated in antigen

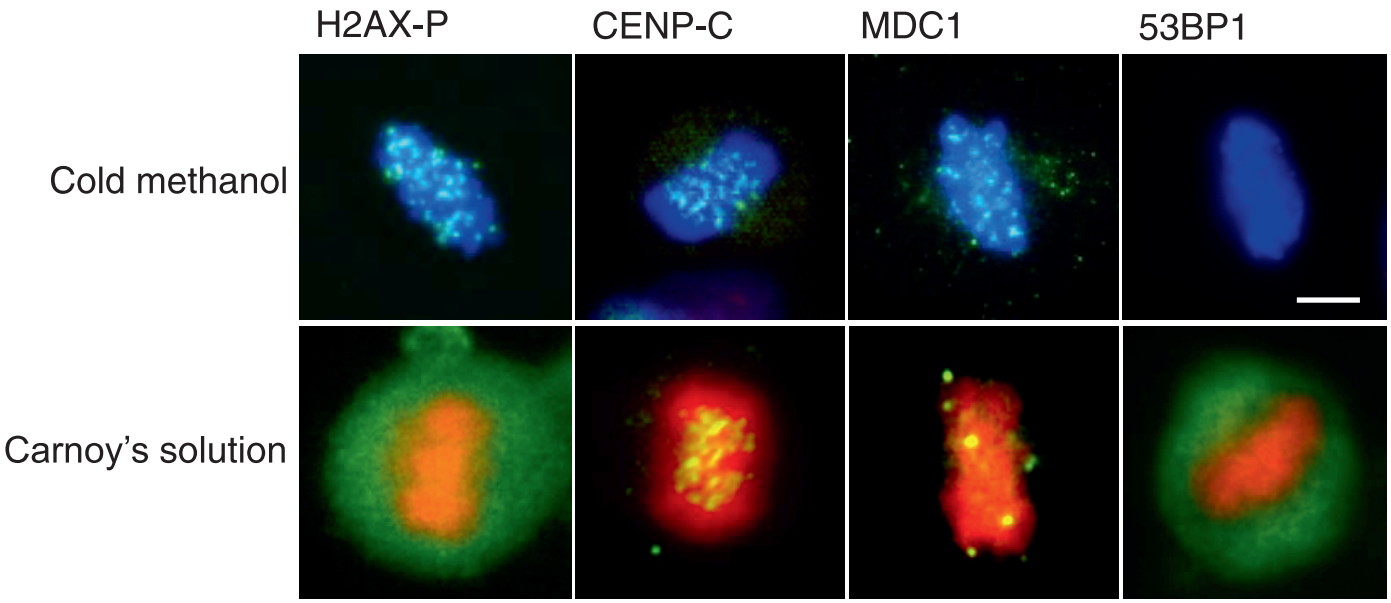

FIG. 1. Reactivity of antibodies in different fixation conditions. Exponentially growing NHDFs were fixed with cold-methanol (upper panels) or with Carnoy's solution (lower panels). Primary antibodies were applied, which were visualized with Alexa Fluor-labelled secondary antibodies. DAPI signals are shown in blue in the upper panels, while they are in red in the lower panels. Bar indicates 5 μm.

retrieval solution (Histofine pH 9.0, Nichirei, Tokyo) prepared with phosphate-buffered saline (PBS), and the samples were immersed for 30 min at room temperature. After three to four washes with PBS, the primary antibodies diluted in TBS-DT (20 mM Tris-HCl, pH7.6, 137 mM NaCl, 0.1% Tween 20, 125 μg/ml ampicillin, 5% skim milk) were treated for 2 h at 37°C, followed by the Alexa Fluor-labeled secondary antibodies for 1 h at 37°C. Then, after extensive washes with PBS, coverslips were mounted on glass slides with 10% glycerol/PBS containing 1 μg/ml of 4′,6-diamino-2-phenylindole (DAPI). The primary antibodies used in this study include rabbit anti-CENP-C antibody (dilution was 1:100, Abcam Japan, Tokyo), mouse anti-phosphorylated histone H2AX antibody (1:100, BioLegend, San Diego, CA), and rabbit anti-MDC1 (1:100, BioLegend, San Diego, CA). The secondary antibodies were goat Alexa Fluor 555-labed anti-rabbit IgG (1:1000, Thermo Fisher Scientific, Tokyo) and goat Alexa Fluor 488-labed anti-mouse IgG (1:1000, Thermo Fisher Scientific, Tokyo).

Image Capturing and DCA

Metaphase images were acquired using a fluorescence microscope (BZ-9000, KEYENCE, Japan) and a microscope (DP70-WPCXP, OLYMPUS, Japan). Captured metaphase images were quantitatively analyzed with Image J software (Rasband WS, ImageJ. National Institutes of Health, Bethesda, MD; 1997–2018 https://imagej.nih.gov/ij/).

To compare the results obtained from CENP-C immunofluorescence with those obtained by Giemsa staining the positions of each cell were recorded when fluorescence images were taken. After Giemsa staining each address was recalled, and the same cells were photographed.

Chromosomal abnormalities were recorded as dicentric or multicentric (three or more centromeres). Chromosome analysis was performed according to the International Atomic Energy Agency (IAEA) criteria (*1, 3*). The metaphase cells with 45 or 46 centromeres were selected, and 100 metaphases of each group were evaluated. The analysis was performed by a trained and experienced observer. Dose-dependent dicentric chromosome induction curve was analyzed using the Dose Estimate software ver. 5.2 (*22*).

Quantitative analysis of CENP-C fluorescence intensities was performed by Image J software, and detailed method was provided in the supplementary figure legends.

Statistical Analysis

Statistical analysis was performed using JMP data analysis software. One-Way ANOVA Kruskal-Wallis test/Steel-Dwass test and trend test were used for groups and doses, respectively. The difference between the CENP-C-DCA and Giemsa-DCA dose-response curves was estimated by the ''compareTwoGrowthCurves'' function in the statmod package in R (*23*), in which the number of permutations was set to 100,000.

RESULTS

NHDFs cultured on coverslips were fixed with cold-methanol or Carnoy's solution, and fluorescence images were obtained from the metaphase cells. As shown in Fig. 1, antibody recognizing phosphorylated histone H2AX, alternatively γ-H2AX, which is frequently used as a surrogate marker for DNA double-strand breaks (DSBs), gave discrete foci in methanol-fixed metaphase cells, however, foci were completely disappeared in those treated with Carnoy's solution. In contrast, antibody against CENP-C showed signals not only in methanol-fixed metaphase cells but also in Carnoy-fixed cells. CENP-C signals were detectable in all four mitotic phases (Supplementary Fig. S2; https://doi.org/10.1667/RADE-22-00050.1.S1). Signals in metaphase cells were also obtained by anti-MDC1 antibody, while there was no signal of 53BP1, which is another DSB marker. Anti-CENP-C antibody was then applied for chromosome spreads prepared from unirradiated metaphase cells fixed with Carnoy's solution (Fig. 2). It successfully detected centromeres on every metaphase

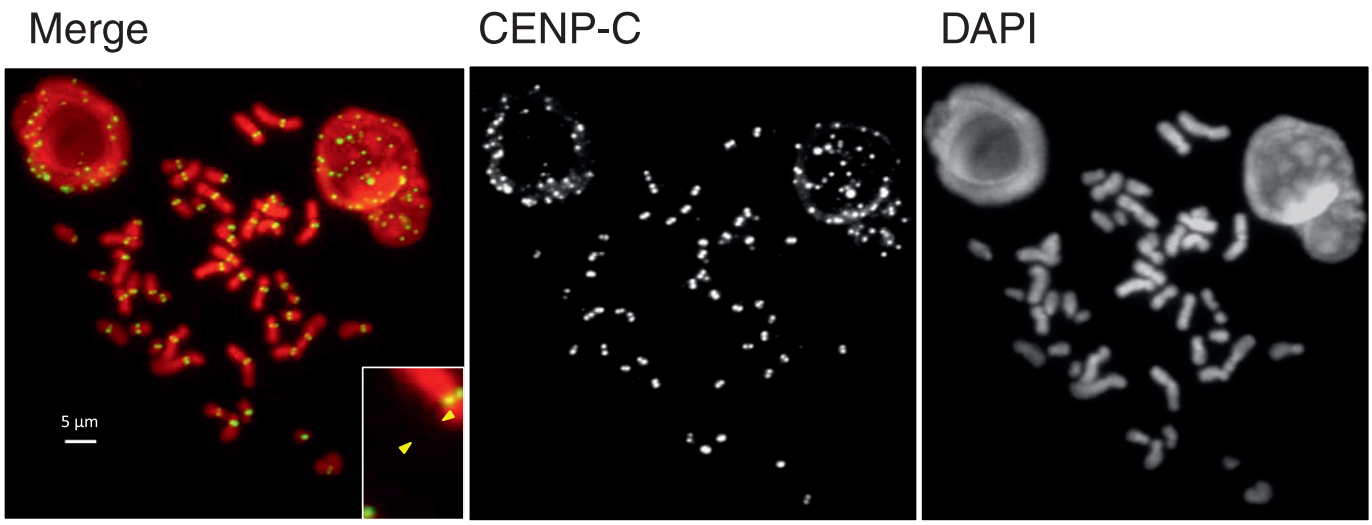

FIG. 2. Visualization of centrosomes by anti-CENP-C antibody in metaphase chromosome spread. Metaphase chromosome spreads prepared using NHDFs fixed in Carnoy's solution were stained with anti-CENP-C antibody (green fluorescence) and chromosomal DNA was stained with DAPI (red artificial color). Left panel; merged image, Middle panel; CENP-C signals, Right panel; DAPI signals. The scale bar represents 5 μm.

chromosome. Doubled CENP-C signals on each sister centromeric region were observed in all 46 human chromosomes.

Dose-dependent induction of dicentric chromosomes were examined in cells exposed to various doses of γ rays. To compare the results with those obtained by the conventional dicentric chromosome assay, metaphase chromosome samples, stained with anti-CENP-C antibody, were observed, and the fluorescence images were memorized with their addresses. Then, the same chromosome samples were re-stained with Giemsa's solution, and the same metaphase chromosomes on the same addresses were photographed. As shown in Fig. 3, the CENP-C staining apparently marked two sister centromeres on dicentric chromosomes induced by 10 Gy, which were also confirmed by Giemsa staining. In addition to dicentric chromosomes, chromosome fragments, which did not have centromeres, were clearly identifiable as chromosomes without any CENP-C signals (Fig. 3). In all cases, dicentric chromosomes identified by two CENP-C signals were perfectly matched to those detected by conventional DCA. On the contrary, some dicentric chromosomes, which were rather difficult to be determined by Giemsa's staining, were thoroughly marked by CENP-C antibody.

Dose-response curves were presented in Fig. 4. Mitotic cells were collected by incubating them for 24 h after various doses of γ rays. The samples were stained with anti-CENP-C antibody, as well as with Giemsa's solution. One-hundred metaphases at each dose were analyzed, and the results were summarized in Table 1. As dose-response curves were plotted in Fig. 4, it was obvious that dicentric chromosomes were induced in a dose-dependent manner.

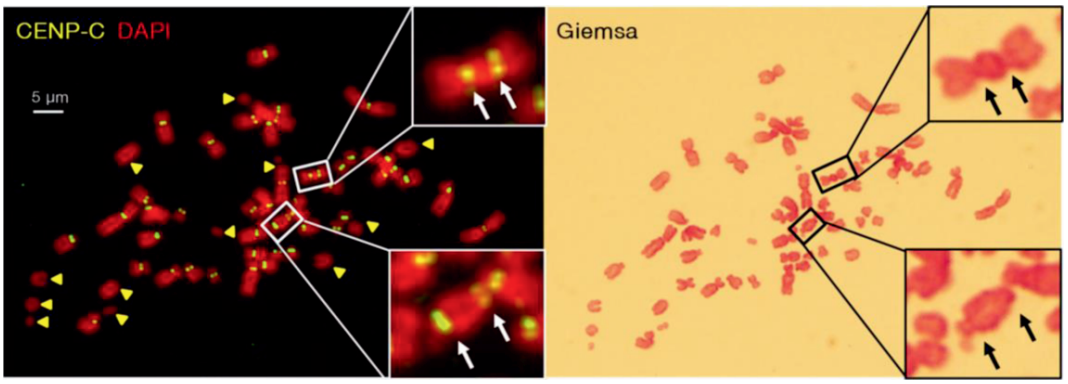

FIG. 3. Detection of chromosome aberrations on the same metaphase chromosome spread. Metaphase chromosome spreads prepared using NHDFs were stained with anti-CENP-C antibody, photographed, and the position of each metaphase was recorded. Then, the chromosome sample was re-stained with Giemsa's solution, and the same chromosome spreads recorded were photographed. In the left panel, yellow color signals indicate centromeres, and white arrows in the insets indicate the position of centromeres. Yellow arrow heads indicate chromosomal fragments without CENP-C signals. In the right panel, black arrows in the insets indicate the position of centromeres on the same dicentric chromosomes. The scale bar represents 5 μm.

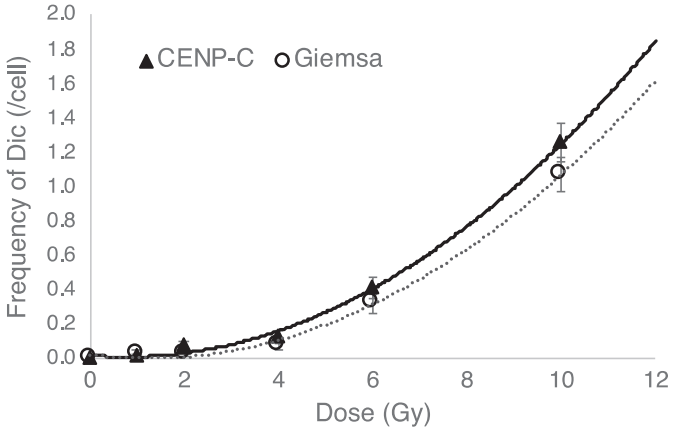

FIG. 4. Comparison of the dose-response cures between samples stained with anti CENP-C antibody and Giemsa's solution. The metaphase chromosome samples prepared using NHDFs were stained with anti-CENP-C antibody, and some of the same samples were stained with Giemsa's solution. One hundred mitosis were counted in each dose group (Table 1), and the dose-response curves were analyzed by Dose Estimate ver. 5.2 software. Obtained equations are as follows. For anti-CENP-C antibody: $Y = 0.0000\ (\pm 0.0000) + 0.0026\ (\pm 0.0098) \times D + 0.0116\ (\pm 0.0017) \times D^2$, $r = 0.9972$. For Giemsa staining: $Y = 0.0000\ (\pm 0.0000) + 0.0036\ (\pm 0.0133) \times D + 0.0093\ (\pm 0.0023) \times D^2$, $r = 0.9940$. Y: yield of chromosome aberrations, D: dose (Gy), r = correlation coefficient. Statistical difference between the dose-response curves for the two methods was tested by the ''compareTwoGrowthCurves'' function in the 'statmod' package in R (*23*), which showed it non-significant ($P = 0.354$).

The results were analyzed using the analysis software, Dose Estimate ver.5.2. For dicentric chromosome evaluation using anti-CENP-C antibody, the equation was $Y = 0.0000\ (\pm 0.0000) + 0.0026\ (\pm 0.0098) \times D + 0.0116\ (\pm 0.0017) \times D^2$, in which Y and D indicate the yield of chromosome aberrations and dose (Gy), respectively. For dicentric chromosome evaluation using Giemsa-stained samples, the equation was $Y = 0.0000\ (\pm 0.0000) + 0.0036\ (\pm 0.0133) \times D + 0.0093\ (\pm 0.0023) \times D^2$. In both cases, high correlation coefficients were obtained (CENP-C: $r = 0.9972$, Giemsa: $r = 0.9940$), demonstrating that two assays were perfectly comparable. Difference between the dose-response curves for the two methods was tested, and there was no statistically significant difference ($P = 0.354$).

As shown in Fig. 3 and Supplementary Fig. S3 (https://doi.org/10.1667/RADE-22-00050.1.S1), we sometimes observed that CENP-C signal on one of the two sister centromeres was greatly diminished in irradiated cells, which was infrequent in unirradiated cells (Supplementary Fig. S3, upper images). Such abnormality was not detectable with Giemsa staining. Since unequal fluorescence signals on the sister centromeres were more profound in cells exposed to higher doses, quantitative evaluation of centromeric fluorescence intensity was performed using image analysis software (Supplementary Fig. S4, and Supplementary Figure Legend; https://doi.org/10.1667/RADE-22-00050.1.S1). In Fig. 5, higher and lower fluorescence intensities from the sister centromeres were presented. It was indicated that the distribution of higher fluorescence signals was much wider in cells received higher doses. In addition, difference in the fluorescence intensities between sister centromeres were more pronounced with radiation exposure, particularly in 4 and 6 Gy irradiated cells (Supplementary Fig. S5; https://doi.org/10.1667/RADE-22-00050.1.S1).

Although our validation study was performed mainly on NHDFs, the CENP-C signals were also detected in the human T lymphoblastic leukemia cells, MOLT-4 (Supplementary Fig. S6; https://doi.org/10.1667/RADE-22-00050.1.S1), indicating that the protocol is applicable to human peripheral blood lymphocytes.

DISCUSSION

While it is general to validate the technique for biodosimetry in human peripheral blood lymphocytes, current study used normal human fibroblasts. This was because we were needed to confirm and evaluate the protocol in many times and in many ways using commercially available antibodies against CENP-C, so that not clinical materials but cultured normal human fibroblast line, which were easily available and easy to handle, was applied. There was also an institutional limit to use clinical human materials. Although we confirmed that the protocol could be applied for human lymphocyte cell line, MOLT-4 (Supplementary Fig. S6; https://doi.org/10.1667/RADE-22-00050.1.S1), studies are still needed to demonstrate whether it is applicable to human peripheral blood lymphocytes.

It is obvious that the introduction of fluorometric assays has greatly improved the sensitivity and credibility of DCA. For example, the Centromere-Telomere FISH is the promising technique to apply for DCA. However, the FISH-based assay uses DNA or PNA probes conjugated with fluorescence dyes, which needs denaturation of chromosomal DNA, and it still needs technical experiences. Therefore, we intended to establish more simple and easier technique without any special technical needs. Since previous studies established immunofluorescence techniques using antibody against one of the centromere proteins, CENP-C (*19*), we decided to apply this method for our evaluation.

Although fluorometric detection of cellular proteins have already been utilized for many purposes in the life science fields, almost all cases utilize common fixatives, such as cold methanol and 4% formaldehyde. In contrast, cytogenetics uses metaphase chromosome samples, prepared using Carnoy's solution, which is the mixture of methanol and acetic acid. The Carnoy's fixative is essential for homogeneous spreading of mitotic chromosomes, but it sometimes removes cytoplasmic protein components (*24–26*) as well as the epitopes recognized by antibodies (*19*). In fact, we demonstrated that antibodies against phosphorylated histone H2AX at serine139 and 53BP1, which are the common antibodies applied for the detection of DSBs, did not show any signal, confirming that the corresponding epitopes were

TABLE 1
Frequencies of Chromosome Aberrations

Dose	No. cells counted	No. dicentric chromosomes	No. cells with dicentric chromosome: No. dicentric chromosomes per cell 0	1	2	3	4	5	6	7	8	Y ± SE
CENP-C												
0	100	0	100	0	0	0	0	0	0	0	0	0.000 ± 0.000
1	100	2	98	2	0	0	0	0	0	0	0	0.020 ± 0.014
2	100	7	93	7	0	0	0	0	0	0	0	0.070 ± 0.026
4	100	13	88	11	1	0	0	0	0	0	0	0.130 ± 0.036
6	100	41	70	20	9	1	0	0	0	0	0	0.410 ± 0.064
10	100	125	48	17	15	11	5	2	0	1	1	1.250 ± 0.112
Giemsa												
0	100	0	100	0	0	0	0	0	0	0	0	0.000 ± 0.000
1	100	3	97	3	0	0	0	0	0	0	0	0.030 ± 0.017
2	100	5	95	5	0	0	0	0	0	0	0	0.030 ± 0.022
4	100	8	92	8	0	0	0	0	0	0	0	0.080 ± 0.028
6	100	32	79	14	4	2	1	0	0	0	0	0.320 ± 0.057
10	100	105	50	18	16	11	2	2	1	0	0	1.070 ± 0.103

degraded or denatured by Carnoy's fixation. Considering that Carnoy's fixative contains acetic acid, it might hydrolyze and distort the structure of proteins, which could conceal the antigenicity of proteins, making immunostaining impossible. Since Carnoy's fixation is essential in order to prepare samples suitable for biodosimetry, it is indispensable to find out centromeric proteins resistant to Carnoy's fixation, and our study and others demonstrated that CENP-C was detectable by antibodies even after the treatment with Carnoy's solution (*19, 27, 28*).

There are several kinds of centromere proteins, such as CENPs, but not many antibodies are commercially available for Carnoy's solution-fixed samples. For example, studies showed the similar results with the CREST antibody (*29*), and antibodies against CENP-C, CENP-B, and CENP-E (*28*). Among them, we applied here an antibody against CENP-C, which was confirmed to retain its antigenicity under Carnoy's fixation (*19*). There was no clear evidence explaining why CENP-C was still recognizable after Carnoy fixation. However, even the antibody against CENP-C, not all antibodies were able to detect CENP-C on metaphase chromosomes, suggesting that specific antibody recognizing specific epitope(s), which are resistant to the fixing action, might be applicable for immunofluorescence method.

The gold standard for biological dosimetry is the analysis of dicentric chromosome after Giemsa staining, but this

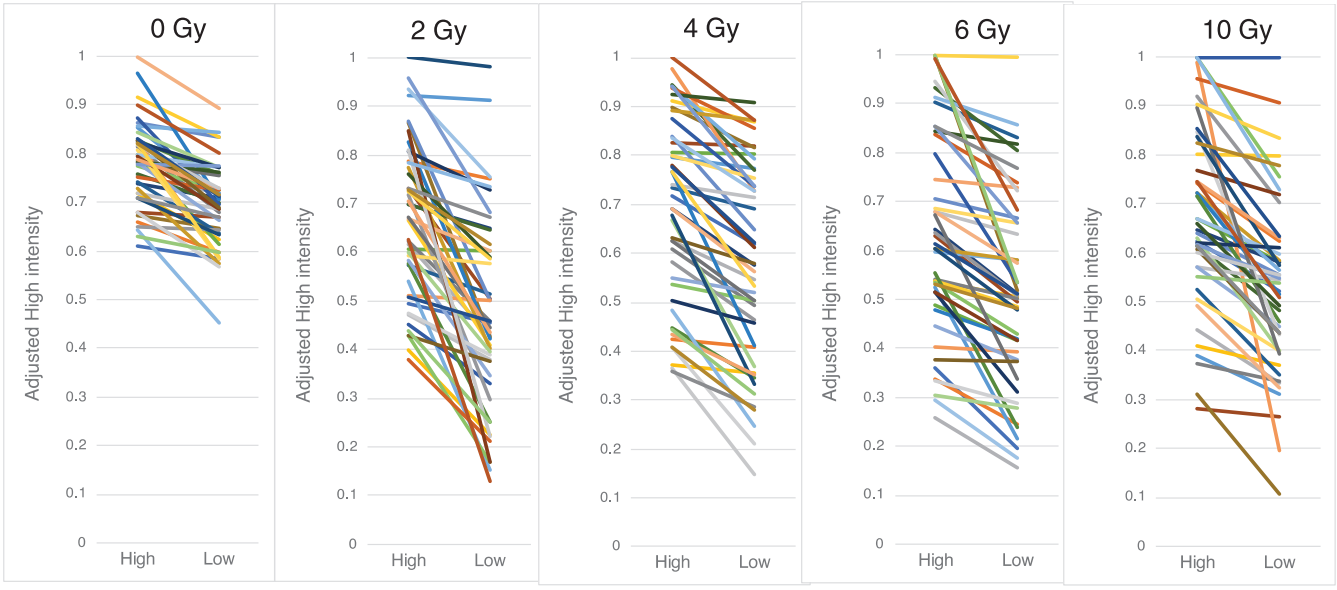

FIG. 5. Quantification of CENP-C signals at the centromeres. Quantification of CENP-C peak fluorescence was performed as described. Difference in the CENP-C fluorescence intensities of the sister centromeres were evaluated in 46 chromosomes within one cell. In each graph, the higher CENP-C signal intensities were indicated on the left, while the lower signal intensities were indicated on the right. One line represents the sister CENP-C signals. Total 46 different lines indicate difference in each sister centromere on 46 chromosomes.

method requires skilled analysis experience and is difficult to calculate for many samples at once. For example, the accident at the Fukushima Daiichi Nuclear Power Plant caused more attention to the health effects stem from low-dose radiation exposure (*30*), however, it was totally impossible to evaluate exposed doses to the public by DCA, since it needed so many metaphase counts to evaluate such low doses. Recently, there has been increasing cases of medical exposure, which also bring anxiety about its health effects. In fact, the increase in DIC was reported by the medical exposure under 100 mSv by computed tomography (CT) (*31*). In any case, it requires many cells to be analyzed for accurate dose estimation, which also requires trained technicians.

The CENP-C-based DCA is reliable, which was confirmed by the comparison between the CENP-C-based assay and conventional Giemsa staining assay. The dose-dependent induction curves were comparable, indicating the usefulness of the method (Fig. 4). Particularly, the CENP-C-based method might visualize the hidden centromeres, which were difficult to identify without any experience and skills. For example, there are two representatives dicentric chromosomes shown in Fig. 3, in which the upper has two obvious centromeres clearly detectable by Giemsa staining, but the lower has no apparent indication of additional centromeres in a Giemsa-stained chromosome. This must be an example where we need trained experiences required for proper analysis of dicentric chromosomes on Giemsa-stained samples, otherwise we might underestimate exposure dose.

Our method is not only useful for DCA assay but also valuable as it provides some insights into previously undescribed effects of radiation on centromeres. As shown in Fig. 5, radiation exposure triggered diversification of the fluorescence intensities of CENP-C signals. While the fluorescence intensity of two CENP-C signals on the sister centromeres in the control cells indicated little or no difference, a more obvious difference between the higher and lower CENP-C signals was existed in irradiated cells, and the difference became more pronounced in those exposed to higher radiation doses. Although the mechanism of heterogeneous CENP-C fluorescence is still to be determined, it was reported that CENP-C was recruited to the sites with DSBs created by laser scissor (*32*), so that it is likely that radiation exposure caused relocation of CENP-C from the centromeres. The biological significance of heterogeneous CENP-C localization is still to be determined, but relocation of centromeric proteins may have some effects on centromere function. In fact, it was shown that CENP-C signals were found predominantly on active centromeres, indicating that centromere function could be regulated by the centromeric proteins (*19, 27–29, 33*). It should be emphasized that the heterogeneous CENP-C localization on the sister centromeres observed here was totally different from the observation in previous studies, which demonstrated homogeneous absence of CENP signals in one of the two centromeres of dicentric chromosomes (*27, 28*), It can be hypothesized that heterogenous DSB induction in or near the centromeric region may affect the localization of centromeric proteins, and this possibility must be examined in future experiments.

In conclusion, our study demonstrated that the anti-CENP-C antibody was applicable to the Carnoy-fixed metaphase chromosome samples and detect radiation-induced dicentric chromosomes. There is an alternative way to prepare chromosome samples for immunofluorescence, which is to use metaphase spread prepared by cytospin after hypotonic treatment (*33*). The method is applicable to CENP-C-based DCA, however, the cytospin is not always available. In contrast, despite that current method still requires a rather expensive fluorescence microscope, it is more common than the cytospin. Furthermore, there was a report indicating that CENP-C-based assay could be applicable to flow cytometry (*34*). In any case, we demonstrated here that the CENP-C-based DCA and Giemsa staining DCA provided equivalent results, indicating that the established method could be used for biodosimetry. The advanced DCA using immunofluorescence with anti CENP-C antibody is expected to be one alternative method for the conventional DCA for biodosimetry in radiological and nuclear accidents.

SUPPLEMENTARY MATERIALS

Supplementary Fig. S1. Dose rate mapping inside the irradiation chamber. Absorbed dose rate data at 15 cm from the center measured with the DoseAce FGD-1000 system are demonstrated (mGy/min). The dose rate for each position was measured five times with 1 Gy (1,000 mGy/min), and data are presented as mean dose rate ± standard deviation. Culture flasks were irradiated in an area with a radius of 5 cm from the center, shown by the dotted line.

Supplementary Fig. S2. Visualization of centrosomes by anti-CENP-C antibody in different mitotic phases. Exponentially growing NHDFs were fixed with Carnoy's solution. Anti-CENP-C antibody was applied, and the signals were visualized with Alexa Fluor-labelled secondary antibody. DAPI signals are shown in red. CENP-C signals were examined in interphase, prophase, metaphase, and anaphase. Bar indicates 5 μm.

Supplementary Fig. S3. Unequal CENP-C signal fluorescence in metaphase chromosomes obtained from irradiation cells. Images were obtained from mitotic chromosome spread prepared from the control NHDFs (upper) and 4 Gy irradiated cells (lower). The samples were stained with anti-CENP-C antibody (green fluorescence) and chromosomal DNA was stained with DAPI (red artificial color). The white arrow heads indicate chromosomes, in which one centromere shows reduced CENP-C signal.

Supplementary Fig. S4. Quantification of CENP-C fluorescence intensities in NHDFs. The green fluorescence signals were separated from the red fluorescence signals.

Then, a line-profile of the image, which was chosen to take the maximum peaks of the fluorescence, was obtained (Supplementary Fig. S3). Then, the peak values and integrated peak densities were quantified for sister centromeres. In some cases (case 2 in supplementary Fig. S3), there was no apparent signal peak obtained, so that the peak position was estimated considering the distance between two sister chromatids. Given the signal distribution of the plot profile, the two highest values, indicated by blue arrows, are obtained from the peak intensity lists. After measuring 45–46 CENP-C sister signals per cell.

Supplementary Fig. S5. Dose-dependent variation of CENP-C fluorescence signals in NHDFs. The difference in the CENP-C fluorescence was summed in each chromosome group, and the results of statistical analysis are shown. The bars and the cross indicate median, the boxes indicate 1st and 3rd quartiles, and points indicate outliers. As a nonparametric test, the Kruskal-Wallis test was performed ($P < 0.05$). Next, the Steel-Dwass test showed significant differences between 0 Gy and 6 Gy, and between 4 and 6 Gy ($P = 0.002$, $P = 0.005$), but the trend test for dose showed no significant differences ($P = 0.1298$).

Supplementary Fig. S6. Visualization of centrosomes by anti-CENP-C antibody in acute T lymphoblastic leukemia cell line, MOLT-4. Exponentially growing MOLT-4 cells were fixed with Carnoy's solution. Anti-CENP-C antibody was applied, and the signals were visualized with Alexa Fluor-labelled secondary antibody. DAPI signals are shown in red. Bar indicates 5 μm.

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