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Persistent *in vivo* cytogenetic effects of radioiodine therapy: a 21-year follow-up study using multicolor FISH

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ABSTRACT

Our previous studies demonstrated the cytogenetic effects in the peripheral blood lymphocytes of a 34-year-old male patient who received ablative radioactive ^{131}iodine therapy (RIT) on two different occasions in 1992 and 1994. Assessment of RIT-induced chromosomal damage by the cytokinesis-blocked micronucleus assay (CBMN) showed the persistence of elevated micronucleus frequency in this patient for more than two decades since the first RIT. Subsequent cytogenetic analysis performed in 2012 revealed both stable and unstable aberrations, whose frequencies were higher than the baseline reported in the literature. Here, we report the findings of our recent cytogenetic analysis peformed in 2015 on this patient using the multicolor fluorescence *in situ* hybridization (mFISH) technique. Our results showed that both reciprocal and non-reciprocal translocations persisted at higher frequencies in the patient than those reported in 2012. Persistence of structural aberrations for more than two decades indicate that these aberrations might have originated from long-lived T-lymphocytes or hematopoietic stem cells. Our study suggests that the long-term persistence of chromosome translocations in circulating lymphocytes can be useful for monitoring the extent of RIT-induced chromosomal instability several years after exposure and for estimating the cumulative absorbed dose after multiple RITs for retrospective biodosimetry purposes. This is perhaps the first and longest follow-up study documenting the persistence of cytogenetic damage for 21 years after internal radiation exposure.

KEYWORDS: *in vivo* exposure, ^{131}Iodine, multicolor fluorescence *in situ* hybridization, chromosomal translocations, micronuclei, telomere and centromere FISH analysis

INTRODUCTION

Cytogenetic assays are the methods of choice for radiation dose assessment when intended or unexpected human exposures occur in the absence of physical dosimetry devices. It has been widely accepted that accurate dose estimation is critical for effectively planning appropriate medical/clinical management for mitigation of illness and severity of radiation injuries. Radiopharmaceutical agents are used widely in the fields of nuclear medicine and radiation oncology. Since its introduction in the 1940s for treatment of hyperthyroidism, the use of radioiodine has increased significantly and currently represents ~90% of all therapies in nuclear medicine. The use of radioiodine warrants further study for evaluation of biological effects prospectively, since it has a short physical half-life (8 days) and an even shorter biological half-life (24 h) and decays by emitting gamma rays and beta particles throughout the body. Patients undergoing therapy with medical isotopes such as ^{131}I provide a unique research opportunity for studying both the acute and chronic biological effects of radiation administered under carefully controlled conditions, allowing prospective follow-up studies for weeks, months, years and even decades after exposure.

Our previous studies [1, 2] demonstrated the cytogenetic effects in the peripheral blood lymphocytes of a 34-year-old male patient

who received ablative radioactive ^{131}iodine therapy (RIT) on two different occasions in 1992 and 1994. Persistence of chromosomal aberrations in circulating lymphocytes of patients following radiation exposure was first reported in 1962 by Bender and Gooch [3]. Studies on atomic bomb survivors showed elevated chromosome aberration rates in the exposed individuals that had persisted for at least two decades after the acute external exposures to thousands of individuals in 1945 [4–7]. The persistent cytogenetic effects were also reported in Chernobyl accident victims in 1986 [8] as well as in the nuclear industry workers in the USA with accidental exposures to plutonium [9–11]. Although persistence of chromosomal aberrations has been reported in persons exposed to high-dose radiation, studies on long-term monitoring of cytogenetic damage following exposure to low doses of medical/diagnostic radiation exposure are very limited. Here, we report a long-term cytogenetic study on a patient over a period of 21 years using multiple assays ranging from the very earliest method (solid Giemsa staining) in the 1950s to the most advanced molecular method (mFISH) developed in the 21st century. Our study demonstrates the persistence of structural chromosomal aberrations in this patient for more than two decades, which is likely due to RIT-induced DNA damage inflicted either in long-lived T-lymphocytes or in hematopoietic stem cells.We suggest that stable structural chromosome aberrations such as translocations are potentially useful for monitoring the long-term effects on chromosomal instability caused by internal radiation exposure.

MATERIALS AND METHODS

Patient history

The patient was a healthy, non-smoking male who was diagnosed with papillary thyroid carcinoma in November 1991 followed by a total thyroidectomy in December 1991 at 34 years of age. The patient is a health physicist by profession and and did not have any history of occupational or medical exposure to ionizing radiation prior to the diagnosis of thyroid cancer in 1991. Prior to medical diagnosis of his pathological condition, the patient actually participated as a donor in a study on assessment of micronuclei (MN) formation after *ex vivo* irradiation with X-rays. This enabled us to determine his baseline MN frequency before the radioiodine treatment. In mid-January of 1992, the patient received 1.78 GBq (48 mCi) of ^{131}I as ablative therapy for residual tumor cells. The MN analysis on this patient was undertaken 11 days after the radioiodine treatment and continued for 14 serial monthly samples, followed by quarterly sampling up to 2 years. Due to a suspicious lesion in the lung after 26 months of radioiodine treatment, the patient was subjected to a second radioiodine treatment with 14.5 GBq (392 mCi) in mid-March, 1994. The MN analysis continued during this period and 6 months after the second treatment. The blood sample collected from this patient in April of 2015 was utilized in the current study for the analyses of MN, dicentric chromosomes and translocations.

Lymphocyte culture and metaphase chromosome preparations

A 10 ml sample of whole blood was obtained from the donors (35 and 55 years of age), and the blood collection was performed with the donor's consent in strict compliance with the Institutional Review Board (IRB) protocol (ORAU000349). Aliquots of the blood sample were cultured for a period of 48 h in RPMI medium supplemented with 2% phytohaemagglutinin (PHA), 10% heat-inactivated fetal bovine serum (Hyclone,GE,USA) and antibiotics, followed by the addition of colcemid (0.1 μg/ml) for the last 24 h. The cells were harvested at 48 h (from the initiation of culture with PHA). Cells were treated with a hypotonic solution (0.56% KCl) for 18 min at 37°C and fixed in three changes of ice-cold acetic acid: methanol (1:3) mixture. An aliquot of fixed cells (25–30 μl) was gently dropped at the center of the glass sides and air-dried for multicolor fluorescence *in situ* hybridization (mFISH) and analysis.

Analysis of micronuclei in binucleated cells

For the analysis of the MN frequency in binucleate cells, peripheral blood lymphocytes were grown in the presence of 2% PHA for 44 h, followed by the addition of cytochalasin B (5 μg/ml) for the last 28 h; the cells were harvested 72 h after culture initiation. Binucleate cells with or without MN were imaged and analyzed using the fluorescence microscope (Carl Zeiss Imager.Z2) equipped with ISIS software (MetaSystems, Boston, MA)

FISH and multi-color fluorescence *in situ* hybridization

The procedure for the fluorescence *in situ* hybridization (FISH) technique using peptide nucleic acid–based human telomeric and centromeric DNA probes was essentially the same as described by the manufacturer (PNA Bio, Newbury Park, CA). For the present study, Tel C, a C-rich telomere probe for the leading strand with repeats of TAACCC and a pan centromeric probe for human alpha staellite repeats (AAACTAGACAGAAGCAT) were used. The mFISH technique was performed essentially as described before [12, 13]. Briefly, slides were treated for 1 min with 0.001% acidic pepsin solution (0.01N HCl) at 37°C for 1–2 min, followed by two washes of 5 min each in phosphate-buffered saline. The slides were post-fixed for 10 min in a solution of formaldehyde/$MgCl_2$ (1% formaldehyde/50 mM $MgCl_2$ in PBS). The slides after denaturation (2X SSC at 70°C for 20 min and after cooling to ambient temperature 1 min in 0.07N NaOH) were dehydrated in a graded series of ethanol (30%, 70%, 90% and 100%) and air dried. The mBAND probe was denatured separately by incubation at 75°C for 5 min, followed by incubation at 37°C for 30 min to allow the annealing of repetitive DNA sequences. An aliquot of the 10 μl probe was placed on the slide and covered with a coverslip. The slides were kept in a humidified hybridization chamber at 37°C for at least 72 h. The unbound probe was removed by washing the slides in pre-warmed (75°C) 1X SSC (pH 7.0–7.5) for 5 min, followed by incubation in 4XSSCT (4X SSC with 0.1% Tween 20) for 5 min. Indirectly labeled probe (Cy5), if needed, was amplified by incubation for 30 min with each of the antibodies. The slides were incubated first with a 1: 50 dilution of Cy5-conjugated streptavidin in blocking buffer (5% non-fat dried milk prepared in 4X SSC with 0.1% Tween 20), followed by a 1:50 dilution of biotinylated anti-streptavidin in blocking buffer and Cy5-conjugated streptavidin (1:50 dilution). All the antibodies were purchased from Invitrogen, Carlsbad, CA. The slides were washed three times for 5 min each in 4XSSCT after incubation with each of the antibodies to remove the unbound

probe. The slides, after a final wash in 1X PBS for 5 min, were air dried at ambient temperature. The nuclei were counterstained with DAPI (Vectashield Laboratories, Burlingame, CA, USA). Metaphase cells were detected by the metaphase finder (MetaSystems, Boston, MA) program, and the mFISH images were captured under a ×63 oil immersion objective lens, using the Zeiss epifluorescence microscope. Image analysis was performed using the ISIS software (MetaSystems, Boston, MA, USA) essentially according to the published procedure. Normal and aberrant chromosomes were identified by unique chromosome-specific processed color generated by the ISIS software based on pixel intensities of combinatorial labeling of the five fluorochromes (FITC, Spectrum Orange, Texas Red, DEAC and Cy5). Translocations (one-way and two-way) were identified by color junctions in 600 well-spread metaphase cells.

Statistical analysis

Data obtained on MN, dicentric chromosomes and translocations are expressed as mean ± standard error of the mean (SEM). The Student paired *t* test was used to analyze the differencs between the means. A *P* value of <0.05 was considered to be significant.

RESULTS

Elevated frequency of structural chromosome aberrations detected by mFISH

Our earlier studies detected the persistence of elevated chromosomal damage in the form of MN, dicentric chromosomes and translocations in a 34-year-old male patient who received ablative ^{131}I therapy on two different occasions in 1992 and 1994 [1, 2]. In this study, we examined the frequency of structural chromosomal aberrations in the same patient 21 years after the second radioiodine exposure by using the state of the art technique, mFISH. Representative pictures of mFISH-labeled aberrant metaphase cells with one- and two-way translocations are shown in Fig. 1. Out of the 600 metaphase cells analyzed, 65 cells (10.83%) were found to be aberrant. Translocations were found in 8.83% of the total cells (53 of 600 cells analyzed), with 57 translocations [57 translocations (reciprocal 31 and non-reciprocal 26) in 600 cells (9.5%)], with a frequency of 0.095 ± 0.012 (mean ± SE) translocations/metaphase cell. Among the 53 aberrant cells with translocations, 4 metaphase cells showed a total of 9 non-reciprocal translocations; two translocations in each of the three cells and three translocations in one cell. Chromosomes 1, 2, 3 and 4 were found to be involved in 36 of the 57 translocations with other chromosomes, accounting for 57.9% of the total yield. It is interesting to note that reciprocal translocations involving chromosomes 2 and 7 were found in 3 cells. Among all the chromosomes, chromosome 4 was involved in greatest number of translocations (11 out of 57 translocations) with other chromosomes, followed by chromosome 10 (10 of 57 translocations), chromosome 2 (9 of 57 translocations) and chromosome 1 (6 out of 57 translocations). Our earlier conventional FISH study using a cocktail of three chromosome-specific probes (1, 4 and 12) revealed a genome equivalent of 41 ± 7 total translocations (inclusive of complete and incomplete exchanges) in 1000 cells, compared with 95 translocations (frequency adjusted for 1000 cells) detected by mFISH

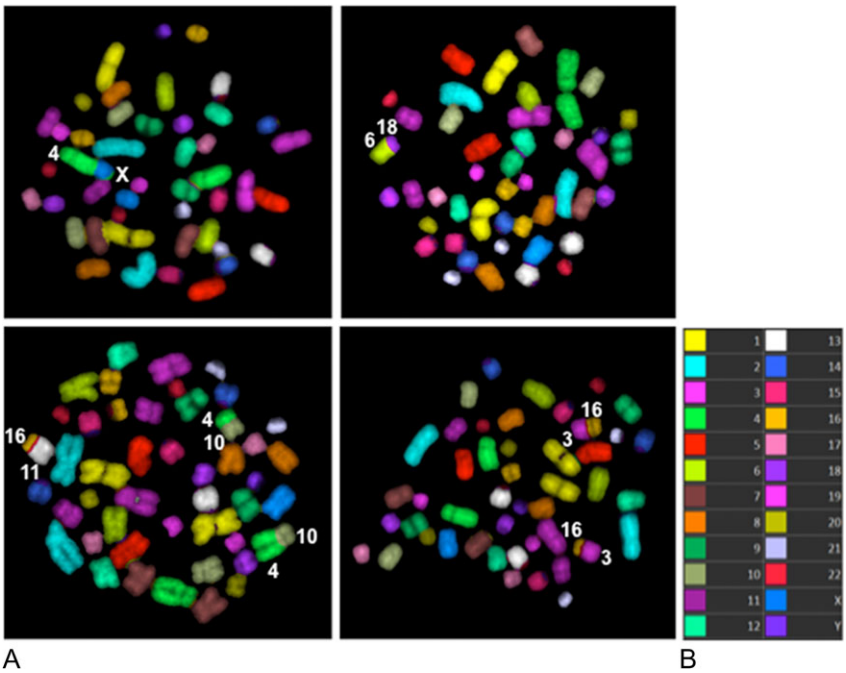

Fig. 1. (A) Representative mutlicolor FISH pictures of aberrant metaphases with one way and two way translocations are shown. The translocated chromosomes are marked with numbers. (B) Chromosomes involved in translocation were identified by pseudocolor patterns generated by ISIS software based on the intensities of five different fluorochromes used for the combinatorial labeling of human chromosomes.

methodology in this study. The frequency of translocations detected by the mFISH technique was significantly higher than that detected by the cocktail of 3 whole chromosome-specific probes. Using the mFISH technique, we found that only 21 of the 57 total translocations involved chromosomes 1, 4 and 12. The frequencies of all types of stable and unstable chromosome aberrations are shown in Table 1.

Elevated frequency of unstable chromosome aberrations

In addition to translocations, three chromatid breaks (chromosomes 2, 5 and X), one chromosome break (Chromosome 4) and 12 acentric fragments (3 of chromosome 1, 2 of chromosome 4, 2 of chromosome 5, 2 of chromosome 20, 1 of each of chromosomes 3, 9 and 19) were observed. Observation of increased frequencies of stable and unstable aberrations suggests an elevated level of chromosomal instability in the radiotherapy patient. The frequencies of all types of stable and unstable aberrations were found to be 0.121/metaphase cell (73 aberrations in 600 cells). A total of 8 hypodiploid (<46 chromosomes) and 12 hyperdiploid (>46 chromosomes) cells were detected during the analysis of 600 metaphase cells by the mFISH technique. Although chromatid breaks, hypo- and hyperdiploidy as well as other chromosome anomalies are not directly induced by radiation, they can be considered as suitable indices for assessing the extent of genomic instability [14, 15].

The dicentric chromosome analysis performed for the first time on the blood lymphocytes of this patient in 2012 (20 years after the first radioiodine *in vivo* exposure) by both conventional Giemsa staining and FISH techniques revealed frequencies of 10 ± 3.2 and 6 ± 1.3 in 1000 metaphase cells, respectively. Since the dicentric chromsosome analysis was not performed until 2011, data on the initial dicentric frequency immediately or after the two radioiodine treatments were not available. Considering the accepted baseline frequency of 1 dicentric chromosome in 1000 cells, it was concluded that the dicentric frequency observed in the radioiodine patient was significantly higher than the background level. However, our recent FISH analysis on the peripheral blood lymphocytes collected from this patient in 2015 (21 years after the two radioiodine therapies) revealed a further elevation in dicentric chromosome frequency (5 out of 251 cells, 0.02 dicentrics/per metaphase cells; 20 per 1000 cells). The representative aberrant metaphases with dicentric chromosomes detected by FISH using human telomere- and centromere-specific peptide nucleic acid (PNA) probes are shown in Fig. 2. Although the precise reason for the elevated dicentric frequency is not known, it may be due to the persistence of dicentric-bearing long-lived lymphocytes, or to the lymphocytes generated from damaged stem/progenitor cells. Alternately, delayed genomic instability triggered by radioiodine exposure can also contribute to an elevation in dicentric chromosome frequency.

Table 1. Stable and unstable chromosomal aberrations observed in the radioiodine-treated patient

Stable aberrations	
Total number of metaphase cells analyzed	600
Normal cells	535 (89.16%)
Aberrant cells	65 (10.83%)
One-way translocations	26 (4.33%)
Two-way translocations	31 (5.16%)
Total translocations	57 (9.5%)
Unstable aberrations	
Total number of metaphase cells analyzed	600
Chromatid breaks	3 (0.5%)
Chromosome breaks	1 (0.16%)
Acentric fragments	12 (2%)
Dicentrics	5[a] (2%)
Rings	1[a] (0.39%)

[a]251 cells were analyzed by FISH using centromere- and telomere-specific PNA probes. A total of 8 hypodiploid (<46 chromosomes) and 12 hyperdiploid (>46 chromosomes) cells were detected during the analysis of 600 metaphase cells by the mFISH technique.

Elevation of micronuclei frequency

The baseline frequency of MN estimated in 1991 was found to be 6.0 ± 1.7 per 1000 binucleate cells (0.6% of cells with MN) for this patient before he was diagnosed with thyroid cancer and subsequently received ablative radioiodine therapy. The MN frequency determined at a monthly interval for 12 months in 1992 after the first radioiodine treatment varied from 0.018 to 0.035/binucleate cell (1.8% to 3.5% of cells with MN). The MN frequency measured at 2-month intervals in 1994 after the second ^{131}I treatment ranged from 0.059 to 0.102/binucleate cell, with a peak value of 0.102 in September of 1994. The highest frequency of MN (0.11/binucleate cell) ever recorded in this patient after the second ^{131}I treatment was in January of 1995. This frequency (0.11) was 18.33-fold more than that of the baseline frequency (0.006) observed in this patient before the radioiodine treatment. Subsequent MN analysis performed from 1995 through 1997 at 3- and 6-month intervals showed small fluctuations, and a MN frequency of 0.073 was observed in January of 1997. The increase in MN frequency caused by the radioiodine treatment was found to be highly statistically significant ($P < 0.05$) when compared with the baseline frequency. MN analysis performed in March 2012 showed a frequency of 0.016/binculeate cell, which was still 2.66-fold more than the baseline frequency. MN analysis performed in April 2015 revealed a similar MN frequency of 11 in 695 binucleate cells (0.015/cell) in the patient relative to 3 MN in 526 binucleate cells cells (0.005/cell) in the sample from a 35-year-old healthy male donor. The MN frequencies (averaged for multiple sampling times in a year) observed over the years from 1991 through 2015 in this patient are summarized in Fig. 3. High frequencies of MN observed after almost 21 years of radioiodine exposure indicates the persistence of chromosomal instability in this patient.

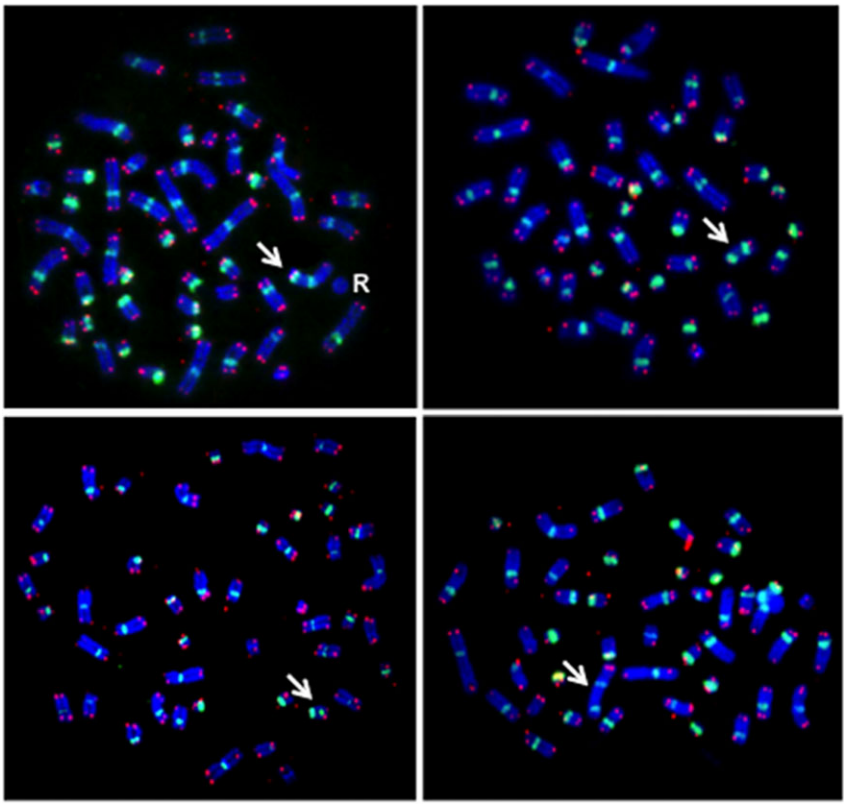

Fig. 2. Detection of dicentric chromosomes in the patient's sample by FISH using human centromere- (green color) and telomere- (red) specific peptide nucleic acid (PNA) probes. Arrows indicate the R-Ring chromosomes with two centromeric spots (green color). The metaphase chromosomes were counterstained with DAPI (blue color).

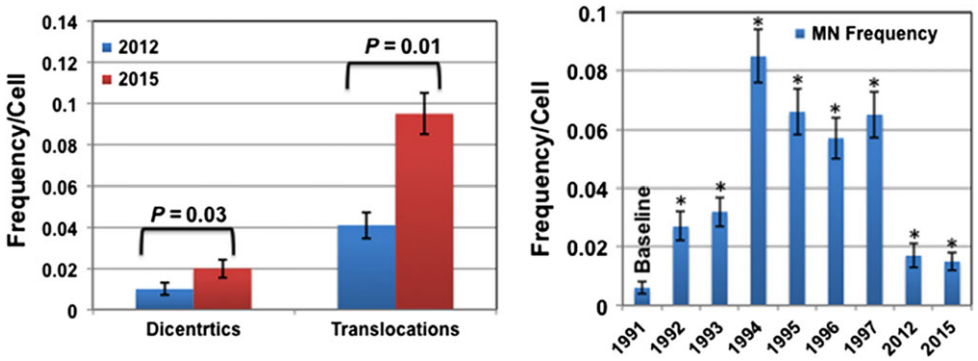

Fig. 3. (A) Frequencies of dicentric chromosomes and translocations detected in the patient blood samples collected in 2012 and 2015. Note that the frequencies of both dicentric chromosomes and translocations are elevated in the sample analyzed in 2015 relative to that of 2012. The translocation frequency detected by mFISH was almost doubled in 2015. (B) Frequency of MN detected in the patient from 1991 (pre-radioiodine treatment) through 2015 (post-treatment) is shown. Bars represent the SE of the mean. Asterisks indicate that the *P* values are statistically significant ($P > 0.05$).

DISCUSSION

Therapeutic application of radioiodine for hyperthyroidism and thyroid cancer in humans provides an unique opportunity for assessing the effects of internal radiation exposure with a feasibility of performing long-term follow-up studies. Earlier reports demonstrated that the frequency of persistent chromosomal damage correlates with absorbed radiation dose in exposed humans [16–18]. Increased frequencies of MN, dicentric chromosomes and translocations were reported by us in a patient 20 years after the *in vivo* exposure to radioiodine [1, 2]. Both stable and unstable

chromosomal aberrations were observed in this patient at higher frequencies than those reported for similarly aged human populations [1, 2]. In this study, we monitored the long-term effects of RIT on the frequency of structural chromosomal aberrations in the entire human genome by the mFISH technique, following two rounds of RITs in 1992 and 1994. The mFISH technique is distinctly advantageous over conventional FISH because it alleviates the need for converting the frequencies to genome equivalents, and therefore eliminates the associated statistical uncertainties, if any, with such a conversion. The mFISH revealed that the frequency of both reciprocal and non-reciprocal translocations was 0.095/metaphase cell, which was 2-fold more than that detected by FISH in 2012 (0.041/metaphase cell), using a cocktail of three chromosome-specific probes. Of interest, mFISH detected the involvement of chromosomes 1, 2, 3 and 4 with other chromosomes in 57.89% of the total translocations. Similar to translocations, the frequency of dicentric chromosomes detected by FISH in the present study was also higher than that detected in 2012. Similar observations have been reported by Puerto *et al.* [19] in 10 radioiodine therapeutic thyroid cancer patients by FISH, using a cocktail of chromosome-specific probes for chromosomes 1, 4 and 10.

Khvostunov *et al.* [20] recently reported the yield of unstable and stable chromosomal aberrations in 24 differentiated thyroid cancer patients who were either treated for the first time or multiple times with radioiodine. In corroboration with our study, frequencies of both stable and unstable aberrations had increased considerably in these patients before and after radioiodine therapy when compared with the baseline frequencies reported in the literature. Using the elevated frequencies of chromosomal aberrations, the authors were able to estimate the cumulative whole-body dose after multiple radioiodine treatments [18]. Using the micronucleus assay, a biodose ranging from 0.089 $mGy.MBq^{-1}$ to 0.197 $mGy.MBq^{-1}$ was reported by a few studies [21–23]. M'Kacher *et al.* [24] reported a mean dose of 0.146 $mGy.MBq^{-1}$ using the chromosome aberration assay, and the activity of ^{131}I was 3.7 GBq in all of these above-mentioned studies. A biodose of 0.23 mGy/MBq was obtained according to the Medical Internal Radiation Dose (MIRD) procedures [25]. On the basis of these values in the literature, we estimate that our patient might have absorbed a whole-body dose in the range of 1.3–2.9 Gy by the second treatment with 14.5 GBq of ^{131}I [2].

^{131}I, due to its emission of gamma rays and beta particles during decay, is extremely hazardous when absorbed into cells and tissues. Considering the half-life of radioiodine, which is ~8 days, it is logical to expect that the absorbed radioiodine would have been completely eliminated from the patient during the follow-up study period, which was more than 21 years from the second administration of radioiodine in 1994. In this study, T-lymphcoytes stimulated by PHA were utilized for the detection of chromosomal damage in the patient. It has been suggested that naïve T cells that frequently circulate between blood and lymph can survive for prolonged periods of time by being in a resting stage, thereby maintaining the immunological responsiveness to new antigens [26–28]. It is currently unclear which T cells (naïve or memory) are preferentially stimulated by PHA, and whether or not long-lived naïve T cells retain the cytogenetic damage owing to their quiescent stage. An equally likely possibility is that the DNA damage initially induced by the radioiodine treatments persisted in a subset of stem/progenitor cells, giving rise to different types of stable and unstable chromosomal aberrations in mature lymphocytes. Observation of clonal translocations in A-bomb survivors suggests that a single stem cell of an adult can generate long-lived myeloid and lymphoid progeny, accounting for a substantial fraction of circulating lymphocytes and hematopoietic progenitors [29, 30]. It is worth noting that no clonal translocations were observed in the current study. Further evidence for the long-time persistence of stable chromosomal aberrations in hematopoietic stem and progenitor cells came from the study of Kreja *et al.* [31], who examined the hematopoietic colonies derived from the peripheral blood samples of 9 highly exposed individuals after 7–25 years of radiation accidents that occurred in Moscow (1971), Kazan (1975) and Chernobyl (1986). Interestingly, no unstable aberrations were detected in this study. Persistence of translocations was also reported in 8 radiotherapy patients (total radiotherapy dose ranged from 40–80 Gy) with different types of cancer over a 5-year period [32]. In an 8-year follow-up study, Natarajan *et al.* [33] did not find any trend for the persistence of translocations, as varying levels of increase and decrease in translocations were found among the radiation accident victims of Goiânia.

The frequencies of dicentric chromosomes and translocations were distinctly higher in the blood sample analyzed in 2015 compared with those analyzed in our earlier study in 2012. We believe that the fluctuations observed in the frequencies of chromosomal aberrations may be due to varying number of damaged cells being present in the circulating blood during the collection times. Alternately, the increased frequency of stable chromosomal aberrations detected by mFISH may also have been due to delayed effects of genomic instability triggered by radioiodine-induced DNA damage accumulation. Further studies are required to clarify whether or not the DNA damage inflicted in the adult stem cells by radioiodine therapy is the root cause for the ensuing chromosomal aberrations at enhanced levels in this patient. The current study indicates that the persistence of stable and unstable aberrations induced by RIT can be of use, not only in long-term monitoring of genomic instability, but also in predicting the absorbed dose by retrospective biodosimetry, as recently demonstrated by Khvostunov *et al.* [20]. However, extreme caution is necessary when extrapolating the initial yield of stable aberrations to equate absorbed radiation dose when the therapeutic radiation exposure is high and non-uniform in nature.

CONCLUSIONS

Our 21-year cytogenetic follow-up study suggests that the internal exposure to gamma rays and β-particles emitted during the decay of radioiodine can cause long-lasting cytogenetic damage in humans, and that the persistence of stable chromosomal aberrations is likely due to the long-lived nature of a subset of damaged T lymphocytes. Our study suggests the possibility that stable chromosomal aberrations can be effectively used for retrospective biodosimetry for internal radiation exposure.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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