Cytogenetic analysis in 16-year follow-up study of a mother and fetus exposed in a radiation accident in Xinzhou, China

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Article history:
Received 19 March 2013
Received in revised form 24 May 2013
Accepted 27 May 2013
Available online 3 June 2013

Keywords:
Radiation accident
Prenatal exposure
Cytogenetic analysis
Fluorescence in situ hybridization
Dose estimation

In November 1992, a radiation accident occurred in Xinzhou, due to the collection by a farmer of an unused 60Co source; 37 individuals were exposed to ionizing radiation. Three individuals died and the farmer's 19-weeks-pregnant wife suffered acute radiation symptoms. Conventional chromosome analysis, cytokinesis-block micronuclei (CBMN) assay and fluorescence in situ hybridization (FISH) painting with three pairs of whole chromosome probes were used to analyze chromosomal aberrations for the pregnant female and her baby during the 16 years following the accident. The yields of dicentrics and rings (dic+r) continually declined between 41 days and 16 years after the accident. The frequency of binucleated MN also decreased over time for both mother and daughter. Sixteen years after exposure, the yields of dic+r and binucleated MN decreased to normal levels, but the reciprocal translocation frequencies remained elevated, for both mother and daughter. FISH results showed a decreasing yield of translocations with time. Based on the changes in maternal translocation frequency, the daughter's dose at the time of exposure was estimated at 1.82 (1.35–2.54) Gy. This was consistent with the clinical manifestations of severe mental retardation and low IQ score. FISH-based translocation analysis can be used for follow-up studies on accidental exposure and, after correction, for retrospective dose estimation for individuals prenatally exposed to radiation.

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1. Introduction

Biological dosimetry using the analysis of dicentrics or micronuclei (MN) in human lymphocytes is a well-established method, especially for acute radiation exposure [1–3]. However, dicentrics or MN decline over time and are not appropriate for dose estimation of exposures that occurred more than one month previously. Fluorescence in situ hybridization (FISH)-based translocation analysis is a useful method for retrospective dose estimation [4]. Recent evidence, however, shows that the persistence of translocation should also be questioned [5,6].

On November 19–27, 1992, a radiation accident occurred in Xinzhou, Shanxi Province, China [7]. In the morning, a farmer, unaware of the hazard, picked up an unused 396 GBq Cobalt-60 source and kept it in his jacket pocket. He was sent to hospital, after complaining of nausea and vomiting later in the afternoon. The jacket with the source remained with him during his hospitalization and altogether thirty-seven individuals were exposed to radiation during this period [8]. He died on December 2, 1992. His father and elder brother, who had been taking care of him at the hospital, both died during the following week.

His wife (“M”) assisted with the care of her husband and was intermittently exposed to the source for several days when she was 19 weeks pregnant. She requested medical assistance in Beijing on December 17, 1992. The doctors suspected that she was suffering from radiation exposure. A peripheral blood sample was collected from “M” for biological dose estimation on December 30, the 41st day after the accident. Her whole-body biological dose was estimated at 2.30 Gy (95% CI: 2.07–2.50 Gy) using lymphocyte chromosome analysis [8]. The equivalent whole-body dose weighted by hematological stem cell survival was estimated to 2.0 Gy and the estimated dose of uterus was 1.4 Gy. Considering the clinical symptoms (fever, hair loss, and bone marrow hypoplasia) and physical and biological dose estimations, “M” was diagnosed...
with the moderate bone marrow form of acute radiation sickness. She recovered after comprehensive treatment with antibiotics, blood transfusion, and hematopoietic growth factor infusion [10].

An ultrasonography examination at 36-week gestational age showed intrauterine growth retardation of the fetus. A baby girl ("B") was born on March 24, 1993 at 73 weeks gestation by artificial rupture of the fetal membranes and labor. The Apgar score for the infant was 10 at both 1 min and 5 min after birth. "B" had little hair, which easily fell out upon touch [11].

The present follow-up studies were performed to monitor the cytogenetic changes in "M" since the 41st day after the accident. The follow-up analysis for "B" had been focused on her physical development during the first seven years after the accident and cytogenetic changes, which have also been analyzed since she was 7.2 years old. The cytogenetic analysis techniques used in the present study included the following: conventional chromosome aberration analysis (dicentric plus centric ring, dic + r); the cytokinesis-block micronucleus (CBMN) assay; and translocation frequency by single color fluorescence in situ hybridization (FISH) painting with three pairs of whole chromosome probes. The dose absorbed at the time of exposure for "B" was also estimated in the present study, when the daughter was 16 years old, using FISH analysis.

2. Materials and methods

2.1. Subjects and clinical data

Clinical follow-up studies on "M" and "B", 16 years after the accident, were previously described [12,13]. Briefly, "M" often felt weak and had frequent colds after she recovered from acute radiation sickness. Her hair turned gray, without apparent hair loss, when she was 32 years old, 9 years after the radiation accident. Her menstrual period was reduced from 5–6 days to 3 days during the 16 years after the accident. Sixteen years after the accident, she was diagnosed with bilateral thyroid enlargement (degree 1). Laboratory studies showed that the levels of triiodothyronine, total thyroxine, free triiodothyronine and free thyroxine were increased while thyrotopin decreased. Antithyroid peroxidase antibody levels were also elevated. These symptoms indicated that she had hypothyroidism and chronic thyroiditis. No apparent abnormalities were found in the major organs. No malignant tumor was detected.

The follow-up study on "B" was focused on physical development during the first 7 years. A small head circumference was found at each follow-up except 4 months (Table 1). She appeared healthy, normal, although she often caught cold until she was 4 years and 7 months old. Her grades in school were poor, particularly in mathematics. She was unable to perform simple addition and subtraction. At 16 years old, when she converses with other people, she can only answer simple questions. The results of the China-revised Wechsler Intelligence Scale for Children test (C-Wechsler) for general cognitive ability showed that verbal, performance, and full-scale IQ scores were 51, 50, and 46, respectively, by which we would classify her as having moderate mental retardation. There is no evidence of other developmental abnormality at age 16.

2.2. Blood sample collection

This work was conducted at the National Institute for Radiological Protection (NIRP), Chinese Center for Disease Control and Prevention. The Ethics Committee of NIRP approved all analyses in the present study. The scope of the study was explained to each subject and written informed consents were obtained. Approximately 2–5 ml peripheral blood was collected at each follow-up, with heparinized syringes or heparinized vacuum tubes (Becton-Dickinson, USA) by venipuncture.

2.3. Chromosome preparation, unstable chromosome aberration analysis and dose estimation

Unstable chromosome aberrations were analyzed for "M" at 41, 53, 73, and 117 days, 7.5 years, and 16 years after the accident. For "B", unstable chromosome aberrations were analyzed at 7.5 and 16 years after the accident.

Approximately 1 ml peripheral blood was divided into two parts and cultured in 5 ml RPMI-1640 culture medium containing 0.2% phytohemagglutinin (PHA, Invitrogen, Carlsbad, USA), antibiotics and 20% fetal calf serum (HyClone, USA), Colchicine (Shanghai Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) was added immediately to a final concentration of 0.04 μM g/ml. The mixtures were incubated at 37 °C for 52 h. The cells were then collected and treated with a hypotoninc solution (0.075 M KCl) for 30 min at 37 °C. Followed by two rounds of fixation with methanol–acetic acid (3:1, v/v). Slides were prepared in ice-cold humid conditions and stained with Gemsa. Dicentrics and rings (dic + r) were counted under a light microscope (1000×, Olympus BX51, Japan).

A dose–effect curve obtained from in vitro irradiation experiments, 30Co γ-rays, 0.5–5 Gy [15] was used to estimate the exposure dose of the victims. The dose–effect–relationship was: Y = 0.035 D + 0.069 D² (R² = 0.998), where Y is the number of dic + r per cell and D is the dose in Gy. Another dose–effect curve, for dose range 0.1–0.5 Gy [15,16], Co–γ-rays, was used to estimate the absorbed dose for the victim of, according to the above equation, the estimated dose was lower than 0.5 Gy. This dose–effect–relationship was: Y = 0.058 + 0.014 D + 0.00038 D² (R² = 0.996), where Y is the number of dic + r per 100 cells, and D is the dose in Gy.

2.4. Micronucleus analysis and dose estimation

CBMN analyses were performed for "M" at the 41st day, 7.5 years and 16 years after the accident. For "B", only the 7.5 and 16 years post-accident analyses were conducted. Approximately 1 ml peripheral blood from each victim was added to 4 ml culture medium containing PHA. After 44 h culture at 37 °C, cytchalasin-B (Sigma, St. Louis, MO, USA) was added to a final concentration 6 μg/ml, and the mixtures were then cultured continuously for another 24 h. The cultured cells were collected and treated with a hypotonic solution (0.075 M KCl) for 2 min at room temperature, then fixed with methanol–acetic acid (3:1). Slides were prepared as described above and stained with Giemsa. Binucleated cells were scored under a light microscope. The numbers of the micronuclein signals were measured.

A micronuclei–dose–effect curve was used to estimate the exposure doses of the victims. The dose–effect–relationship was as follows: Y = 1.791 × 3.383 D + 42.88 D² (R² = 0.997), where Y is the number of micronuclei per 1000 cells, and D is the dose in Gy. The dose range could be estimated from 0.1 to 5 Gy [16]. Another dose–effect–curve for the dose range from 0.1 to 0.5 Gy [16] was used to estimate the absorbed dose for the victim if the estimated dose, according to the above equation, was lower than 0.5 Gy. The dose–effect–relationship was: Y = 14.99 + 38.43 D (R² = 0.995), where Y is the number of micronuclei per 1000 cells, and D is the dose in Gy.

2.5. Fluorescence in situ hybridization, translocation analysis and dose estimation

FISH-based translocation analysis was carried out at 7.5 years and 16 years after the accident for "M“. Analysis was performed for “B” only at 16 years after the accident.

Approximately 3 ml peripheral blood from "M" and "B" was used to isolate the lymphocytes with Ficoll–Paque (Amersham Pharmacia Biotech). Cells were washed three times with fresh RPMI 1640 medium (Invitrogen, Carlsbad, USA), and chromosome preparations were prepared by adding colchicine at the start of culturing, according to the method described in Section 2.3. A few drops of cell suspension in fixative were dropped onto dried, alcohol-pre-cleaned slides. The slides were then placed above a container filled with heated water for 20–60 s, depending on the ambient humidity [17]. The remaining cell suspension was kept at −20 °C.

Each slide for FISH was pretreated with RNase A (100 μg/ml) in 2× SSC, Boehringer Mannheim, USA) and proteins K (Sigma–Aldrich, Santa Clara, USA) and fixed in 1% formaldehyde according to the method of Pinkel et al. [18]. The slides were denatured with 70% formamide and 2× SSC at 70 °C for 1–2 min, followed by dehydration by an ice-cold ethanol series (70%, 90% and 100%). The denatured probes, which were labeled with FITC (FITC, Roche, Kumamoto, Japan), were hybridized at 37 °C overnight. Three post-hybridization washes were performed at 42 °C in 50% formamide and 2× SSC for 5 min each, followed by three washes in 0.1× SSC at 50 °C. Chromosome counterstaining was performed with 0.15 μg ml−1 4,6-diamidino-2-phenylindole (DAPI). The slides were stored in a dark –20 °C freezer before analysis.

The FISH slides were viewed with a Zeiss Axioplan 2 Imaging fluorescence microscope (Zeiss, Oberkochen, Germany) with a cooled charge-coupled device (AxioCam HRM, Zeiss) and were analyzed using his FISH analysis software (MetaSystems, Germany). Two-color (green and blue) images were captured, merged and stored. Two thousand cells were scored for each sample.

Metaphases were considered suitable for analysis only if they appeared to be completely bivalent, the fluorescence was bright, the centromeres were morphologically detectable and all three pairs of chromosomes appeared somewhere in the cell. Generally, the scoring criteria followed the conventional scoring system. Each translocation was checked by two observers. The cells were scored as normal when all signals on the three pairs of chromosomes were complete. A reciprocal translocation was recorded if more than six chromosomes were observed with green color and two chromosomes were observed with green-blue color. The FISH results were converted into the genome translocation frequency using the Lucas formula Fx₁/Fx₀ = 2.05 (Fx₀ – 1) / [Fx₀ – 4], where Fx₀ is the translocation frequency detected by FISH, Fx₁ is the full genome translocation frequency, and Fx₀ is the fraction of the genome hybridized, which is 22.3% for chromosomes 1, 2 and 4 of female [19].

A dose–effect–curve obtained during FISH-based translocation analysis was used to estimate the exposure doses of the victims. The dose–effect–relationship was as follows: Y = 0.0037 + 0.0002 D + 0.043 D² (R² = 1.000), where D is the dose in Gy for the genome translocation frequency Y. The dose range could be estimated from 0.5 to 5 Gy [20].
Table 1
The comparison of growth and development index between normal child and 'B'.

<table>
<thead>
<tr>
<th>Age</th>
<th>Height (cm)</th>
<th>Weight (kg)</th>
<th>Head circumference (cm)</th>
<th>Chest circumference (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>95% CI</td>
<td>B</td>
<td>95% CI</td>
<td>B</td>
</tr>
<tr>
<td>Birth</td>
<td>46.4–53.0</td>
<td>44.0</td>
<td>2.5–4.0</td>
<td>2.0</td>
</tr>
<tr>
<td>2 months</td>
<td>54.6–63.6</td>
<td>53.0</td>
<td>4.4–7.1</td>
<td>4.2</td>
</tr>
<tr>
<td>4 months</td>
<td>59.9–68.5</td>
<td>60.0</td>
<td>5.6–8.7</td>
<td>5.5</td>
</tr>
<tr>
<td>14 months</td>
<td>74.3–86.1</td>
<td>78.0</td>
<td>8.2–12.7</td>
<td>9.5</td>
</tr>
<tr>
<td>3.8 years</td>
<td>95.5–110.7</td>
<td>96.0</td>
<td>12.8–20.1</td>
<td>13.0</td>
</tr>
<tr>
<td>4.7 years</td>
<td>101.3–118.5</td>
<td>102.0</td>
<td>13.8–22.9</td>
<td>16.0</td>
</tr>
<tr>
<td>7.5 years</td>
<td>106.2–128.7</td>
<td>123.0</td>
<td>15.5–26.8</td>
<td>19.0</td>
</tr>
<tr>
<td>16 years</td>
<td>151.0–167.1</td>
<td>157.0</td>
<td>41.4–56.7</td>
<td>48.0</td>
</tr>
</tbody>
</table>

* 95% CI, 95% confidence interval of normal Chinese from Ref. [14].

2.6. Statistical analysis

Statistical analyses were performed using Statistical Program for Social Sciences 13.0 software (SPSS 13.0, SPSS Inc., Chicago, IL, USA). The data were expressed as the mean ± standard deviation for chromosome aberration frequency or mean (95% confidence intervals) for estimated doses. All reported P values were two-sided, and a significance level of 0.05 was used as the cutoff for significance.

3. Results

3.1. Dicentrics plus centric rings analysis and dose estimation

Table 2 shows the yields of dic + r in the peripheral blood samples from the two victims. For the “M”, the dic + r frequency gradually declined. On the 117th day after the accident, dic + r yields were reduced to 56% of the levels on the 41st day after the accident, which was the first dic + r analysis after the accident. The relationship between the duration and dic + r frequency fit well to a logarithmic function with the equation \( Y = -0.072 \ln X + 0.657 \) (\( R^2 = 0.9388 \)), where \( Y \) is the dic + r frequency per cell and \( X \) is the time after the accident in days. The average half-lives of the dic + r frequency drops were 415 days. The estimated doses for each time-point also decreased with time. The relationship between the time after the accident and the estimated absorbed dose also fit well to a logarithmic function with the equation \( Y = -0.375 \ln X + 3.59 \) (\( R^2 = 0.9819 \)), where \( Y \) is the estimated absorbed dose in Gy, and \( X \) is the period after the accident in days. For “B”, the dic + r frequency and the estimated dose at 16 years after the accident had both decreased and approached normal levels.

3.2. CBMN analysis and dose estimation

Table 3 shows the results of the CBMN analysis. The micronucleus frequencies in the peripheral blood samples from the two victims decreased with time. For “M”, the MN frequency in binucleated cells changed from 220 per thousand at 41 days to 30 per thousand at 16 years after the accident. The MN frequency in binucleated cells for “B” dropped from 34 per thousand at 7.5 years to 9 per thousand at 16 years after the accident. The estimated dose at each time-point also declined with time.

3.3. FISH-based translocation analysis and dose estimation

Table 4 shows the results of FISH-based translocation frequency and retrospective dose estimation for the victims at 7.5 and 16 years after the accident. For “M”, the genome translocation frequency and the estimated dose also decreased with time, falling by approximately 33.9% over an 8.5-year-period. This reduction, however, was less than that of dic + r. The estimated dose with the residue translocation frequency determined by FISH at 16 years after the accident for “B” was also obtained.

3.4. Estimation of the absorbed dose in utero for “B”

To estimate the in utero absorbed dose for “B” at the time of exposure, the changes of the estimated doses for “M” over 16 years were calculated. Compared to the estimated dose by FISH after 16 years and the biological doses by dic + r analysis at 41 days after the accident, the dose correction factor of the translocation values for the delayed blood sampling at 16 years was 3.03 (2.25–4.24). The estimated in utero dose for “B” at the time of exposure was 1.82 (1.35–2.54) Gy.

4. Discussion

In the present study, three cytogenetic methods, conventional chromosome aberration analysis, the CBMN assay, and FISH-based translocation analysis, were adopted to analyze the radiation-induced genetic changes over time for victims. The yields of all three end-points decreased with time. The absorbed dose for “B” at the time of exposure in utero was also estimated. This is the first study to retrospectively estimate the biological dose for an individual victim who was exposed to accidental prenatal radiation.

Scoring of radiation-induced dic + r has been performed as the “gold standard” of the accidental biodosimetry assessment, and the analysis is normally performed within a month after irradiation [21]. This is not the case if the retrospective dose assessment or follow-up study is carried out due to the continuous loss of dic + r yield. In the present study, at the 117th day after the accident, the dic + r yield for “M” gradually decreased to 56% of the initial levels.
measured at 41 days after the accident. This result is in good agreement with the 50% decrease observed at 100 days after a γ-ray accident [22]. According to our data and the results in the literature, the dic + r yield declined over time after the accidental exposure. The follow-up study on victims at the Goiânia radiation accident in Brazil showed that the average disappearance half-life of the dic + r yield was 130 days [23], which was shorter than the value of 415 days in the present study. It is likely that variation in the half-life of dic + r chromosome aberration exists among different individuals.

Translocation analysis by FISH has been suggested as an assay for retrospective dosimetry because a translocation could pass successively through mitosis and transfer into the daughter cells, persisting long enough to measure doses received months or years before blood sampling [24]. The results of the present study showed that the dose for “M” estimated using dic + r was lower than that determined by the FISH method, 7.5 years after the accident (0.66 Gy vs. 1.15 Gy). This difference also occurred at 16 years after the accident. It is likely that the FISH-based translocation analysis could be the best biological indicator for follow-up studies on radiation exposure. The results of the FISH analysis for “M” showed that the level of reciprocal translocations also declined over time. The study by Camparoto et al. showed a need for appropriate correction factors that consider both the persistence of chromosomal translocations over time and the influence of factors on the inter-individual variability in cellular response to radiation [25]. In the present study, retrospective dose estimation by FISH at 16 years after the accident determined an average correction factor for “M” of 3.03. This factor was calculated by comparing the initial estimated dose with dic + r at 41 days and the estimated FISH dose with the residue translocation at 16 years after irradiation. This factor is similar to the result of the follow-up study on the Goiânia radiation accident, 8 years after the accident [26].

The mean genome translocation frequency of “B” at 16 years after the accident was 0.023 translocations per cell, which is a relatively high incidence. This is not consistent with the findings of A-bomb survivors exposed in utero, which showed no increase of chromosome translocations 40 years after exposure [27]. The difference might result from the time duration after the exposure and from clonal expansion of cells carrying the same chromosome translocations [28]. Clonal expansion is not easy to test by the single-color FISH method used in the present study, and this problem might be solved by multicolor-FISH [28] or G-bandig analysis.

To estimate the absorbed dose for “B” at the time of exposure in utero, the same correction factor was used, considering that both “M” and “B” were exposed to the same radiation. The estimated dose for “B” at the time of exposure in utero was 1.82 (1.35–2.54) Gy, which was lower than that of “M”. This difference results from the shielding effect of the mother’s abdomen and the protection of the womb from radioactive sources outside the mother’s body. The estimated dose for “B” might be inaccurate because the physical growth speed of “B” during gestation was very high and the amount of bone marrow increased rapidly. Thus, the number of bone marrow cells with translocation would have been decreased and the translocation frequency in peripheral blood lymphocytes would be lowered by undamaged cells entering circulation. The true exposed dose for “B” might have been higher than that estimated in the present study.

The studies on survivors exposed in utero to atomic-bomb radiation in Japan showed that the possibility of health effects of radiation on the fetus depends both on fetal gestational age at the time of exposure and the amount of radiation to which the fetus is exposed [29]. Large radiation doses to the fetus during the most sensitive stages of development (between weeks 8 and 15 of pregnancy) can cause birth defects, especially to the brain. Between the 16th week of pregnancy and birth, radiation-induced health effects (besides cancer) are not commonly observed, unless the fetus received an extremely large dose of radiation [30]. At this dose level, the mother could be showing signs of acute radiation syndrome. The clinical findings of “B” included severe mental retardation, low IQ scores, and poor school performance. All of these effects were well in agreement with the estimated absorbed dose (1.82 Gy in average) and the gestational age (19 weeks after ovulation). The estimated absorbed dose of 1.82 Gy was far higher than the threshold doses established from the Japanese atomic bomb study for exposures at weeks 16–25 postconception, which were 0.9 Gy for mental retardation and 21 IQ points decline per Gy (95% CI = 13–30) [31].

5. Conclusion

Three types of cytogenetic analysis were performed during the 16-year follow-up study of a mother and her daughter who were exposed to accidental irradiation in Xinzhou, China. Radiation-induced dicentric plus centric rings or micronucleus yields were eliminated over time. FISH-based translocation frequency also declined, but remained higher than those of dic + r yields at the corresponding time point. The absorbed dose for an individual prenatally exposed was retrospectively estimated according to the dose correction factor calculated from the mother.

Conflict of interest statement

None.
Acknowledgements

The authors wish to thank both accident victims for their contribution to the present study. We also thank Renee Zhao, who provided linguistic support. This work was supported by the National Natural Science Foundation of China (No. 30870749, 81172593).

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