Linear dose response of acrocentric chromosome associations to gamma irradiation in human lymphocytes

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Abstract
Purpose The frequency of acrocentric chromosome associations (ACA) was studied to determine the possible dose–response relationship of gamma irradiation in human lymphocytes.

Methods Peripheral blood collected from three healthy donors was irradiated with 0, 0.1, 0.25, 0.5, 0.75, 1, 2, 3, 4, and 5 Gy of gamma radiation. Chromosomal preparations were made after 48 h of culture as per standard guidelines. The experiment was repeated three times, with a different donor each time.

Results The ACA frequency in irradiated lymphocytes increased with radiation dose. The D–G type of association was most prominent and showed a significant dose-dependent increase in frequency. The dose response of ACA frequency to radiation was found to be linear: ACA frequency = 0.2923 (±0.0276) + 0.1846 (±0.0307) × D (correlation coefficient r = 0.9442). As expected, dicentric chromosome (DC) frequencies followed the linear quadratic fit model, with DC frequency = 0.0015 (±0.0013) + 0.0220 (±0.0059) × D + 0.0215 (±0.0018) × D^2 (correlation coefficient r = 0.9982). A correlation curve was prepared for ACA frequency versus DC frequency, resulting in the regression equation y = 1.130x + 0.4051 (R^2 = 0.7408; p = 0.0014).

Conclusion Our results showed an increase in ACA frequency in irradiated lymphocytes with an increase in radiation dose; thus, ACA may serve as a candidate cytogenetic biomarker for radiation biodosimetry.

Keywords Biomarker · Cytogenetics · Gamma rays · Nondisjunction, genetic · Biodosimetry

Acrocentric chromosome associations (ACA) have been widely studied for their incidence in diverse health conditions like Down’s syndrome [1, 2], mental retardation [3], reproductive disorders [4, 5], aging [6], smallpox vaccination [7], asthma [8], Alzheimer’s disease [9], and oral squamous cell carcinoma [10]. Epidemiological findings suggest that low radiation exposure can be a possible cause of nondisjunction in humans; however, there are few scientific studies to provide strong experimental evidence for this observation. It was reported that exposure to a low dose of radiation produces mitotic nondisjunction in human lymphocytes [11]. The authors observed that the abnormal segregation was not only induced in irradiated cells but also in cells that were incubated with irradiated cell-free plasma or serum, and chromosomes 21 and X were found to be most susceptible to nondisjunction [11]. Later it was suggested that radiation could cause an increase in satellite associations through the aging phenomenon [12].

Ionizing radiation is responsible for induction of DNA double-strand breaks (dsb) and, consequently, chromosomal aberrations are produced. It has been reported that intersatellite fibers or connectives of short arms in acrocentric chromosomes consist of nucleoprotein B, and it was further identified that these chromosomal regions contain DNA complementary to rRNA, i.e., ribosomal DNA [13]. Recently, linkages between rDNA on heterologous chromosomes were revealed by super-resolution microscopy and suggested that the dynamic DNA loci contribute to corporal inter-chromosomal networks that are an essential and a persistent character of genome organization [14]. Chromosomal associations involving 2–5 acrocentric chromosomes were noticed in uranium miners [15]. Significantly higher rates of ACA and predominant occurrence of D–G-
type associations were reported in occupationally exposed workers and spaceflight astronauts [16–18].

Chromosome aberrations and micronuclei frequencies are the most popular cytogenetic parameters used for estimation of absorbed dose in the case of radiation accidents [19, 20]. The dicentric chromosome assay (DCA) was initially designed in 1962 and is now considered the gold standard of radiation biodosimetry by the International Atomic Energy Agency and the World Health Organization (WHO) due to its high specificity and neutrality toward gender, age, or region [21, 22]. Several biodosimetry laboratories are now functional and networked within different countries and regions. However, it is necessary to note that the DCA technique needs well-trained manpower and is time consuming [22–24]. Further, in the event of a large-scale radiation emergency, DCA alone will be difficult for medical management of victims. Therefore, alternate triage model methods are necessary along with DCA.

ACA represent early chromosomal instability/damage, merit attention, and have been advocated as an indicator of chronic ionizing radiation exposure, especially to low doses, based on studies of occupationally exposed persons [17, 18]. Therefore, examining their significance as a candidate cytogenetic biomarker for radiation biodosimetry, the present study was designed to assess the frequency of ACA in human metaphases induced by gamma irradiation and to construct a dose–response curve.

**Materials and methods**

**Sample collection**

Whole blood obtained from three healthy male donors was used to study variations in the frequencies of ACA and dicentric chromosomes (DC) in terms of radiation dose via chromosome preparation. The study was approved by the institutional ethical committee (IEC/38/Research/17). The scope and significance of the study were explained to each donor before sample collection and informed consent was obtained. The donors were 32–55 years old with no history of smoking, chronic disease, viral infection within the past 6 months, or exposure to toxic chemicals or radiation. The heparinized vacutainer tubes were used to collect approximately 10 ml of peripheral blood by venipuncture from each donor. The experiment was repeated three times, with a different donor each time.

**Irradiation**

Blood samples were aliquoted (1 ml each) into 10 cryovials in a biosafety cabinet. The samples were then exposed to a $^{60}$Co $\gamma$-rays teletherapy unit (Bhabhatron II; Panacea Medical Technologies, Bangalore, India). They were irradiated with 0 (sham irradiation), 0.1, 0.25, 0.50, 0.75, 1, 2, 3, 4, and 5 Gy, with dose rate 0.38 Gy/min, source-to-surface distance (SSD) 120 cm, and field size 20 x 20 cm$^2$. Immediately after irradiation, all samples were brought back to the blood culture laboratory and kept at 37°C for 2h to allow DNA repair, as per standard practice in all biodosimetry laboratories for DC and cytokinesis-block micronuclei assays [22].

**Culture setup**

After 2h, lymphocyte cultures were initiated using the established standard method [22, 23]. Briefly, cultures were set up by pouring 8 ml culture medium RPMI 1640 (GIBCO; Thermo Fisher Scientific, Waltham, MA, USA) into a T25 flask and adding 100 μl antibiotic Penstrep (GIBCO). This was then supplemented with 2 ml fetal bovine serum (GIBCO, final concentration 20% v/v) and gently mixed. Thereafter, 1 ml of whole blood was inoculated into the medium and mixed, immediately followed by addition of 400 μl phytohemagglutinin (PHA-M, GIBCO) and incubation at 37°C for 48h. After 24h, 200 μl colchicine (Sigma, Merck, Burlington, MA, USA) with a final concentration of 0.02 μg/ml was added to arrest metaphase and samples were re-incubated at 37°C for another 24h. Following centrifugation, the pellet was suspended in 0.075 M KCl and kept for 25 min at 37°C (hypotonic treatment), again centrifuged, and fixed in chilled Carnoy’s fixative (1 part acetic acid:3 parts methanol). Aliquots of each cell suspension were made and stored at 4°C. The cell suspension was dropped onto precleaned, chilled slides and air-dried. Slide staining was performed with 4% Giemsa stain solution (pH 6.8 phosphate buffer) for microscopic observations.

**Scoring of slides**

**ACA scoring**

The coded slides were scored for the occurrence of ACA following the criteria of Zang and Black [25]. Briefly, the chromosome associations were considered:

1. If the distance between centromeres of the chromosomes was less than the length of the long arm of the largest G chromosome.
2. Longer distance was accepted when short arms were attached through visible threads.
3. If the associating chromosomes were heading towards each other with longitudinal congregation, then a larger distance was accepted (up to the length of the long arm of the largest D chromosome).
**DC scoring**

The standard DCA guidelines of the International Atomic Energy Agency [22] were followed:

1. A complete metaphase with 46 chromosomes, all with a single centromere.

2. If metaphase contained unstable aberrations (dicentric) and was accompanied by an acentric fragment, yet the total count remains 46.

3. Excess acentric fragments not linked with a dicentric or centric ring that increased the chromosome count past 46.

4. Each tricentric corresponds to two dicentrics, accompanied by two acentric/fragments.

### Table 1 Number of metaphases scored for ACA and DCA

| Radiation dose (Gy) | Metaphases scored for ACA | Metaphases scored for DCA |
|---------------------|---------------------------|---------------------------|
|                     | First experiment | Second experiment | Third experiment | Total | First experiment | Second experiment | Third experiment | Total |
| 0                   | 180              | 200              | 200              | 580   | 1000            | 500              | 500              | 2000 |
| 0.1                 | 220              | 200              | 320              | 740   | 500             | 520              | 500              | 2020 |
| 0.25                | 180              | 200              | 200              | 580   | 500             | 500              | 500              | 1540 |
| 0.5                 | 200              | 200              | 180              | 580   | 1000            | 520              | 800              | 2320 |
| 1                   | 240              | 200              | 180              | 620   | 1000            | 520              | 520              | 2040 |
| 2                   | 120              | 160              | 120              | 400   | 500             | 350              | 1000             | 1850 |
| 3                   | 140              | 120              | 160              | 420   | 500             | 520              | 520              | 1540 |
| 4                   | 140              | 120              | 160              | 420   | 500             | 520              | 520              | 1540 |
| 5                   | 160              | 140              | 120              | 420   | 500             | 500              | 500              | 1320 |
| Total               | 1760             | 1740             | 1840             | 5340  | 6500            | 4790             | 7380             | 18670 |

Metaphases from each dose level were analyzed under 1000 X magnification using oil immersion on Axioskop 2 Plus microscope (Carl Zeiss AG, Oberkochen, Germany)

ACA acrocentric chromosome associations, DCA dicentric chromosome assay

### Table 2 Dose versus frequency for ACA and DCA in metaphases of gamma-irradiated lymphocytes

| Radiation dose (Gy) | ACA | DCA |
|---------------------|-----|-----|
|                     | Total metaphases scored | Number of ACA | ACA frequency | Total metaphases scored | Number of DC | DC frequency |
| 0                   | 580 | 79  | 0.14±0.02 | 2000 | 1 | 0.005±0.005 |
| 0.1                 | 740 | 202 | 0.27±0.02 | 2020 | 12 | 0.006±0.002 |
| 0.25                | 580 | 201 | 0.35±0.02 | 2020 | 20 | 0.01±0.002 |
| 0.5                 | 580 | 259 | 0.45±0.03 | 1540 | 31 | 0.02±0.004 |
| 0.75                | 580 | 302 | 0.52±0.03 | 2320 | 56 | 0.02±0.003 |
| 1                   | 620 | 411 | 0.66±0.03 | 2040 | 114 | 0.06±0.005 |
| 2                   | 400 | 277 | 0.69±0.04 | 1850 | 216 | 0.12±0.008 |
| 3                   | 420 | 358 | 0.85±0.05 | 1540 | 384 | 0.25±0.02 |
| 4                   | 420 | 392 | 0.93±0.05 | 2020 | 852 | 0.42±0.02 |
| 5                   | 420 | 447 | 1.06±0.05 | 1320 | 906 | 0.69±0.03 |

Frequency represents ACA/cell, DC/cell; each value represents mean ± SE (standard error)
The Student’s t-test was used for statistical comparison using GraphPad version 8 (GraphPad Software Inc., CA, USA)
Radiation-exposed groups were compared with the sham-irradiated group
ACA acrocentric chromosome associations, DCA dicentric chromosome assay, DC dicentric chromosomes
A minimum of 100 metaphases for ACA and 500 metaphases for DC, from each dose level, were analyzed under 1000 X magnification using oil immersion on an Axioskop 2 Plus microscope (Carl Zeiss AG, Oberkochen, Germany).

The number of metaphases evaluated for ACA and DCA are represented in Table 1. The number of metaphases evaluated per sample for ACA was between 120 and 320, and the total number of metaphases scored was 1760, 1740, and 1840 for the first, second, and third set of experiments, respectively. Thus, a total of 5340 metaphases were scored for ACA. The number of metaphases evaluated per sample for DCA was between 300 and 1000; the total number of metaphases scored was 6500, 4790, and 7380 for the first, second, and third set of experiments, respectively. Thus, a total of 18,670 metaphases were scored for DCA. The scored data were entered into score sheets. After completion of scoring, all slides were decoded and a dose–response curve was constructed. The linear fit model was followed for the dose–response curve of various types of ACA and the linear quadratic fit model for DC using DoseEstimate_v5.3 [26].

**Results**

The ACA frequency in metaphases of human lymphocytes irradiated with 0, 0.1, 0.25, 0.5, 0.75, 1, 2, 3, 4, and 5 Gy of gamma radiation showed a significant increase with increasing dose (Table 2). Representative metaphase spreads showing different types of ACA in gamma-irradiated lymphocytes are shown in Fig. 1. The frequencies of various types of associations observed in metaphases of human lymphocytes exposed to different doses of gamma radiation are presented in Fig. 2.

The D–G type of association was most prominent in all the doses studied and showed a significant dose-dependent increase at each data point. The D–D type of association showed a dose-dependent increase with exception of the 2 Gy data point, and significance was observed only at 3 and 5 Gy radiation doses. The G–G type of association showed a dose-dependent increase except at 0.75 and 4 Gy radiation doses, where a decrease compared to the previous dose was noticed. A significant difference to the sham-irradiated group was observed at 0.5, 0.75, 2, 3, 4, and 5 Gy. The 2D–G type of association showed a dose-dependent
increase except at 2 and 3 Gy radiation doses; however, the increase was significant at higher doses, i.e., 1, 2, 4, and 5 Gy. The 2G–D type of association showed a significant increase only at 2 and 4 Gy radiation doses. No significant difference was observed for the 3G type of association. Likewise, no significant difference was observed for the 3D type of association except at 5 Gy. A significant difference was observed for the 2D–2G type of association only at 4 and 5 Gy radiation doses. However, a significant difference was observed for all types of association except 3G at a 5 Gy radiation dose. The dose–response curves of the various types of ACA and total ACA observed in metaphases of human lymphocytes exposed to different doses of gamma radiation are presented in Figs. 3 and 4.

A parallel but independent assessment of DC was also carried out for the same slides. Representative metaphase spreads showing normal, dicentric, ring, and tricentric chromosomes in gamma-irradiated lymphocytes are depicted in Fig. 5. The DC frequency in metaphases of human lymphocytes irradiated with 0, 0.1, 0.25, 0.5, 0.75, 1, 2, 3, 4, and 5 Gy gamma radiation showed a significant increase with increasing dose (Table 2). The dose–response effect between DC frequency and radiation dose follows a linear model fit up to radiation dose 0.75 Gy; thereafter, it follows the linear quadratic model. Therefore, the linear quadratic model fit was used for DC to construct a dose–response curve (Fig. 6). The correlation curve was prepared for ACA frequencies (y-axis) versus DC frequencies (x-axis) in human lymphocytes (Fig. 7), resulting in the regression equation $y = 1.130x + 0.4051$ ($R^2 = 0.7408; p = 0.0014$).

**Discussion**

Various structural chromosomal alterations are induced by exposure to ionizing radiation, mainly due to DNA double-strand breaks (dsb) and misrepair that ultimately produce dicentric chromosomes, chromosome end fusion, micronuclei, and nucleoplasmic bridges. The incidence of associations between the short arms of acrocentric chromosomes was reported as satellite associations long ago [27–29]. It has been shown that on a per-cell basis, the most frequent associations take place between D and G chromosomes, due to their greater number of possible combinations [30]. The number of associations per cell noted were D–G = 0.91, D–D = 0.37, and G–G = 0.28. It was observed that chromosomes 21 and 22, which are smaller than chromosomes 13, 14, and 15, possess more extended nucleolus organizer regions; consequently, acrocentric chromosomes 21 and 22 enter into associations more frequently than chromosomes 13, 14, and 15 [31]. The background frequency reported for ACA is between 11.65 and 13.21% [27] and 7.84–12.8 associations/cell [16, 32]. It has also been reported that there is no correlation between association frequency and sex, race, or age [33]. However, recent cytogenetic literature is extremely limited—also in the context of our focus on biodosimetry—because of the high degree of skill required for DC detection and the time-intensive analysis. Further, although image-processing algorithms progressing in various laboratories, but the uncertainties and error still bothers for automation.
Fig. 3 Dose–response curve of various types of acrocentric chromosome associations observed in metaphases of human lymphocytes exposed to different doses of gamma radiation. a D–D-type associations; b D–G-type associations; c G–G-type associations; d 2D–G-type associations; e 2G–D-type associations; f 3D-type associations; g 3G-type associations, h 2G–2D-type associations
In the present study, the frequency of ACA showed a significant increase with an increasing dose of radiation. The D–G type of association was predominantly recorded in all doses studied and showed a dose-dependent significant increase. In a cytogenetic study of uranium miners from the Western Carpathians, a wide array of chromosomal aberrations were observed, the most frequent being chromosomal associations involving 2–5 acrocentric chromosomes [15]. Associations between chromosomes 13, 14, 15, and 22 with triradial formations were prominent. Yadav and Seth [16] showed significantly higher rates of ACA: a predominant occurrence of D–G-type associations was noted, while the occurrence of 3D-type associations was least in workers who were occupationally exposed to X-rays. Around a 2.5-fold increase in the frequency of ACA in occupationally exposed hospital workers was recorded [17]. Recently, significantly higher satellite association was observed in astronauts who had participated in a spaceflight [18].

In the present study, the frequency of ACA in metaphases of human lymphocytes irradiated with 0, 0.1, 0.25, 0.5,
Fig. 6  Dose–response curve of dicentric chromosome frequencies in human lymphocytes using the linear quadratic model fit. Frequency = 0.0015 (±0.0013) + 0.0220 (±0.0059) × D + 0.0215 (±0.0018) × D². Weighted chi-squared = 19.8500, degrees of freedom = 7, p-value for goodness of fit = 0.0059; p-values for coefficients (z-test): p_A = 0.2662, p_alpha = 0.0071, p_beta = 0.0000. Correlation coefficient r = 0.9982

Fig. 7  Correlation curve of acrocentric chromosome association (ACA) frequencies versus dicentric chromosome (DC) frequencies in human lymphocytes

0.75, 1, 2, 3, 4, and 5 Gy of gamma radiation showed an increase with increasing dose. The dose–response effect between ACA frequency and radiation dose followed a linear fit. Only a few studies are available on the dose–effect relationship between ACA and radiation exposure. The effect of low-dose X-irradiation on acrocentric chromosome satellite associations was first studied and showed that irradiation may influence the composition of the satellite association complexes [34]. In another study, an increase in acrocentric association with an increase of radiation dose was reported for 0–1 Gy radiation exposure; however, exposure in the range of 1–4 Gy showed an inverse relationship to dose [35], which contradicts our observations in the present study, where an increase in ACA frequency with radiation dose was noticed. It was noticed that at certain data points, ACA subtypes showed a decrease at a higher dose compared to a lower dose, e.g., the D–D type of association showed a lower frequency at 2 Gy (0.15 ± 0.05) than 1 Gy (0.17 ± 0.02), the G–G type of association had a lower frequency (0.09 ± 0.02) at 4 Gy than at 3 Gy (0.10 ± 0.03), and the 2D–G type of association showed a lower frequency at 2 Gy (0.057 ± 0.004) than at 1 Gy (0.07 ± 0.008). However, the total number of ACA and D–G-type associations showed a significant increase with an increase in radiation dose at each data point. In the present investigation, total ACA as well as D–G-type associations emerged as potential cytogenetic parameters for radiation biodosimetry. Regarding this aspect, ACA can be considered for image-based automatic analysis, similar to DC; the complexity is expected to be less in comparison to DC.

A gold standard of radiation biodosimetry, i.e., DCA [22], was used successfully for dose assessment in Chernobyl for post-accident medical management. The strong correlation with physical dosimetry attracted the various organizations and was ultimately considered the gold standard for biodosimetry. This was used subsequently in all radiation accidents including Goiania, Fukushima, and even Mayapuri, Delhi [36–38]. This is routinely in practice for suspected overexposures in occupational workers. However, the DCA technique needs well-trained manpower and is time consuming, which have proved to be the main hindrances to its triage biodosimetry [22–24]. Alternatively, although the IAEA suggests using micronuclei, the analysis is influenced by various factors and micronuclei are induced by several agents; thus, it is not radiation specific [39–42]. It has also been established that at a very low dose range, the DC frequency is very low and becomes extremely difficult and time consuming for analysis [43–47].

In recent years, progress has been made in complete automation of DCA for radiation emergencies through proper validation and optimization by the combined use of image processing and machine learning techniques [48–53]. The latest study by Alsbeigh et al. [54] highlighted the ob-
servation that DC is underestimated in accidental radiation exposures with doses of less than 1 Gy, wherein lies an impending risk of developing late stochastic effects [55]. Since it is a fact that physical inter-chromosomal connections in ACA are the first to break during genomic instability [14], the parameter captured our attention, and the results of the present analysis indicate that ACA can serve as a precise and sensitive tool for generating biodosimetry curves for ionizing radiation exposure. In microscopic analysis, when compared to DC scoring, ACA scoring requires a lower number of total cells; this is an advantage, as a maximum of 100 cells need to be scored for ACA scoring. Further investigations are warranted for multicentric studies and inter-laboratory comparisons for validation of ACA as a sensitive, prospective, cytogenetic biodosimetric marker. Our study is preliminary but provides potential inputs. A machine learning approach for ACA scoring is not yet available. It is interesting to note in the present study that ACA frequency and linear dependency appears similar to gamma H2AX assay, where the number of foci of gamma H2AX per cell increases linearly only up to 1 Gy or 2 Gy [56]. The disadvantage of gamma H2AX is that it is short lived, making triage difficult. Understanding of the linear dose response of ACA will require mechanistic studies.

**Conclusion**

Our results showed an increase in ACA frequency in irradiated lymphocytes with an increase in radiation dose; thus, ACA may serve as a candidate cytogenetic biomarker for radiation biodosimetry. In microscopic analysis, when compared to DC scoring, ACA scoring requires a lower number of total cells, which may be considered an advantage. Further, image-based automatic analysis could be relatively easy to develop and thereby represent another possible perspective of the present study.

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**Author Contribution** RMS and NKC designed the study. RMS and PG generated and analyzed the data, and wrote the manuscript. All authors have reviewed and approved the final version of the manuscript.

**Conflict of interest** R.M. Samarth, P. Gandhi, and N.K. Chaudhury declare that they have no competing interests.
