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Research Article

Assessment of Cytogenetic Abnormalities by FISH in Lymphocytes from a Victim Accidentally Exposed to Cobalt-60

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Abstract:

***Background*:** A radiation accident occurred on 14 June 2011 in an industrial facility in Bulgaria with a cobalt source (137 TBq) that was used to sterilize equipment. Five people received doses exceeding 1Gy, which led to the development of acute radiation syndrome. Biological dosimetry based on dicentric analysis was performed and the average acute whole-body doses estimated for the five patients ranged from 1.2 to 5.6 Gy. The purpose of this study was to evaluate induced chromosome aberrations *in vivo* four months after the accident in the most severely exposed subject.

***Methods*:** Fluorescence plus Giemsa (FPG) staining and Fluorescent *in situ* hybridization (FISH) were used to detect chromosomal aberrations in peripheral blood lymphocytes from the study subject four months after accidental exposure. FISH was performed using DNA probes specific for whole chromosomes 1, 4, and 11.

***Results*:** The mean frequency of dicentrics per cell ± SE was 0.425 ± 0.042, determined by FPG. According to FISH analysis, the genomic frequency of apparently simple translocations (ASTs) per cell was 0.678 ± 0.032. Incomplete translocations accounted for approximately 35% of all translocations. For apparently simple dicentrics (ASDs), the genomic frequency was

calculated as 0.182 ± 0.017. The frequency of complete plus incomplete dicentrics was 0.250 ± 0.019. Chromosome 1 was found to be involved in 52.35% of translocations. Chromosome 4 was found to be involved in 30.20% and chromosome 11 was involved in 22.82% of the total yield of translocations detected. Among 157 aberrant cells, 28 cells (or 17.83%) exhibited one or more complex exchanges.

***Conclusions*:** This is a pilot study for assessing cytogenetic abnormalities *in vivo* in a person from Bulgaria who was accidentally exposed to radiation. It is not possible from this study to relate the high frequency of translocations with a long-term biological effect, but it is a well-known fact that ionizing radiation increases the risk of cancer in some individuals.

Keywords

Chromosome aberrations; radiation accident; FISH analysis

1. Introduction

In June 2011, a severe irradiation accident occurred involving cobalt-60 (Co-60) with high activity, affecting five victims. The accident happened during a recharging operation in an industrial irradiation facility. The withdrawn active tube contained 137 TBq of Co-60, and the personnel exposure time was estimated to be about 25-30 minutes during which they manipulated the tube and circulated it in the room.

The first medical examination of personnel occurred 40 hours after exposure where prodromal signs and symptoms were recorded. Blood samples were taken for differential blood counts and cytogenetic analysis for biodosimetry. These have been reported in separate publications [1, 2]. The patients were treated in Paris with bone marrow growth factors.

The aim of this study was to evaluate *in vivo* by fluorescence *in* situ hybridization (FISH) the spectrum of induced chromosome aberrations four months after the accident in the most affected patient. Just after the accident,in our lab was estimated a whole body dose of 5.60 Gy (95% confidence interval 4.94-6.25) with an initial frequency of dicentrics of 0.96 ± 0.061 [1]. Conventional and molecular cytogenetic methods were applied to detect chromosome damage after accidental exposure to ionizing radiation.

2. Materials and Methods

2.1. Lymphocyte Culture and Slide Preparation

For the present study, a blood sample was taken from the victim four months after the radiation accident.

Peripheral blood lymphocytes were cultured for 50 hours in RPMI 1640 (Sigma-Aldrich) medium supplemented with 15% fetal bovine serum (Sigma-Aldrich) and 100 µg/ml Penicillin–Streptomycin (Sigma-Aldrich), and stimulated with 2% phytohemagglutinin (Gibco-Life Technologies). To be sure that exclusively first division cells would be analyzed, 0.05 mg/ml 5-Bromo-2-DeoxyUridine (BrdU)(Sigma-Aldrich) was added to the culture medium. Cultures were incubated in vented flasks

at 37 °C and 5% CO_2 for 48 hours, and 0.1 µg/ml Colcemid (Invitrogen) was added for the final 2 hours of culture.

Harvesting of the cells was performed according to standard techniques with exposure to 75 mM potassium chloride followed by fixation in 3:1 methanol to acetic acid (Merck) solution. Cell metaphases were then spread onto clean, wet slides. These slides were used for dicentric and FISH assays.

2.2. Cytogenetic Methods

One set of slides was stained using the FPG technique described in the International Atomic Energy Agency (IAEA) report [3]. It is necessary to distinguish between the first and second mitoses. To differentiate chromosomes from cells in their first division from chromosomes that have passed a second or later cell division, 0.5 mg/mL of Hoechst 33258 (Sigma-Aldrich) was applied to the slides. The slides were then maintained for 60 minutes under ultraviolet exposure. After washing, chromosome staining was performed with 4% Giemsa (Sigma-Aldrich).

Fluorescence *in situ* hybridization was performed using XCyting DNA Probes (MetaSystems), following the manufacturer's standard protocol. Chromosome paint mix labeled with fluorochromes specific for whole chromosomes 1, 4, and 11 were applied. As a result, chromosome 1 was painted in green and chromosomes 4 and 11 were painted in red. Nuclear DNA was denatured by incubating the slides at 75° C (+/-1 ° C) for 2 minutes, followed by hybridization at 37° C overnight in a ThermoStat plus denaturation and hybridization system (Eppendorf).

Post-hybridization washes including 0,4x saline sodium citrate solution for 2 minutes and 2x SSC with 0.05% Tween-20 (AppliChem) for 30 seconds at room temperature was performed. Final counterstaining with DAPI (Sigma-Aldrich) (1.5 µg/mL in Vectashield) (4,6-diamidino-2-phenylindole-2-hydrochloride) was applied.

2.3. Scoring Criteria and Chromosome Aberration Classification

Conventional Giemsa staining was done and dicentrics, centric rings, and acentrics were scored only in complete first division metaphases containing 46 chromosomes. For this study, 240 metaphases were analyzed by two independent observers. Metaphase spreads were analysed using the IAEA and ISO guidelines [3].

FISH analysis was carried out manually under an Olympus microscope with 1000x magnification. In addition, a computerized image analysis system with a camera was used to capture and confirm all aberrations. Only metaphases containing approximately 46 chromosomes and all three painted chromosome pairs were analyzed.

Classification of aberrations was also carried out using the modification of the PAINT nomenclature proposed by Tucker et al. [4]. The descriptive terminology of PAINT enables a rapid, reproducible description, even of the most extensively rearranged chromosomes, by classifying each abnormal painting pattern individually [5]. A modification of PAINT criteria, allowing also for mechanistic aspects, would be most advantageous for practical application [5]. We described the observed chromosomal aberrations using the following letters: A and a describe DAPI counterstained chromosomes, B and b describe the two pairs of chromosomes painted in red, and C and c describe chromosomes painted in green. Capital letters describe the chromosome parts

with the centromere. Small letters describe the chromosome parts without the centromere. Using this nomenclature, all cells containing simple aberrations (including ASTs and ASDs) were recorded. Exchange aberrations involving three or more breaks in two or more chromosomes were classified as complex [6]. Because of a very high frequency of complex aberrations, observed chromosomal aberrations were also recorded using conventional nomenclature. A bicolor chromosome with a single centromere in the painted part accompanied by a bicolor chromosome with a single centromere in counterstained part was classified as a complete translocation [7].

Since FISH analysis of bicolored aberrations detects only a portion of the total aberrations present in a cell, the exchange frequency was converted to a whole genome equivalent using the formula derived by Lucas et al. [8]: Fp = 2.05 fp(1-fp)FG, where Fp is the frequency of translocations detected by FISH, fp is the painted fraction of the genome, and FG is the genomic aberration frequency. Chromosomes 1, 4, and 11 comprise approximately 19% of the human genome. Female human chromosome 1 comprises 8.15%, chromosome 4 comprises 6.29%, and chromosome 11 comprises 4.46% of the human genome. Because two colours were utilized for painting the chromosomes (chromosome 1 was green and 4 and 11 were red), we applied $Fp/FG = 2.05[f_1(1-f_1) + f_{4+11}(1-F_{4+11}) - f_1f_{4+11}]$ [3].

3. Results

Four months after the accident, a blood sample was taken from the most affected person to estimate the frequency of radiation-induced chromosome alterations and their persistence over time. FISH and FPG staining were applied.

3.1. Giemsa Staining

A total 240 metaphases was analysed by solid Giemsa staining. One hundred and two dicentrics were observed, and 9 of them were rings. The mean frequency of dicentrics per cell ± SE was 0.425 ± 0.042.

3.2. Fluorescent in Situ *Hybridization*

A total 662 metaphases was analysed in FISH stained preparations The total number of apparently simple translocations and apparently simple dicentrics was 149 and 40, respectively, plus 3 rings (Table 1). Due to the combination of chromosomes 1, 4, and 11, the observed values were converted to full genome equivalent (FG) with a factor for female 0.332 [8]. The calculated genomic frequency of apparently simple translocations per cell using the modification of PAINT nomenclature was 0.678 ± 0.03, or approximately 678 translocations per 1000 cells (Table 2). From these, about 418 translocations were the two-way type. The mean percentage of incomplete translocations was 35%, and similar results were reported by others (Table 3) [9, 10].

The total number of apparently simple dicentrics was 40, and 3 rings were found (two on chromosome 1 and one on chromosome 4). The genomic frequency of ASDs was calculated as 0.182 ± 0.017, or approximately 20 dicentrics per 100 cells. From these, about 12 dicentrics were two-way (Table 2).

Table 1 Simple aberrations.

CELL DESCRIPTION	AST		ASD	
	t(Ca)t(Ac)	44	dic(CA)ace(ac)	10
	t(Ca) ace(c)	0	dic(CA)ace(c)	2
	t(Ac)	19	dic(CA)	2
	t(Ca)	2	dic(BC)ace(bc)	0
	t(Bc)t(Cb)	5	dic(BC) ace(b)	2
	t(Bc)ace(b)	0	dic(BC)ace(c)	0
	t(Cb)ace(c)	0	dic(BA)ace(ab)	16
	t(Cb)	4	dic(BA) ace(b)	3
	t(Bc)	3	dic(BA)	5
	t(Ba) t(Ab)	43		
	t(Ba) ace(b)	3	r(C) ace(c)	2
	t(Ab)	19	r(B) ace(b)	1
	t(Ba)	7		
Total		149		40 +3 rings

Table 2 Chromosome aberrations detected by FISH stained preparation.

Aberration	Cells analysed	Aberrant cells	Normal cells	Total Aberrations	Frequency	SE	Genomic Frequency	SE
Giemsa staining								
Dicentrics+ rings	240	63	177	102	0.425	0.042	-	
FISH staining								
AST	662	121	541	149	0.225	0.02	0.678	0.032
Two-way T	662	88	574	92	0.139	0.01	0.418	0.025
Complete T	662	88	574	102	0.154	0.02	0.464	0.026
Total T	662	125	537	157	0.237	0.02	0.714	0.033
ASD	662	35	109	40	0.060	0.01	0.182	0.017
Two-way dic	662	26	633	26	0,039	0.01	0,118	0,013
Complete dic	662	37	622	42	0,063	0.01	0,191	0,017
Total dic	662	46	613	55	0,083	0.01	0,250	0,019
Insertions	662	14	648	16	0.024	0.01	0.073	0.010
Complex abberations	662	28	634	33	0.050	0.01	-	

AST, apparently simple translocation;dic, dicentrics; T, translocation.

Table 3 Chromosome aberrations detected.

CONVENTIONAL NOMECLATURE	
Stable aberrations	
Total number of metaphase cell analysed	662
Complete translocations	102 (65%)
Incomplete translocations	55 (35%)
Total traslocations	157 (100 %)
Unstable aberrations	
Complete dicentrics	42 (72, 4%)
Incomplete dicentrics	13 (22, 4%)
Rings	3 (5, 17%)

Conventional nomenclature was also applied in part to describe in more detail the entire range of aberrations, especially complex exchanges (defined as at least three breaks in two or more chromosomes). By conventional nomenclature, we observed 102 complete translocations from 157 total translocations with genome frequencies of 0.464 ± 0.03 and 0.714 ± 0.03 per cell, respectively (Table 2). The total amount of complete plus incomplete dicentrics was 55 or converted to FG (full genome frequency) was 0.250 ± 0.02, or approximately 26 dicentrics per 100 cells. Notably, by solid Giemsa staining, we found the frequency of dicentrics to be 0.425 ± 0.042 or 42.5 dicentrics per 100 cells, which is higher than the genome frequency reported by FISH.

Chromosome 1 was found to be involved in 78 of the 149 ASTs with other chromosomes, accounting for 52.35% of the total translocations detected. Chromosome 4 was found to be involved in 45 translocations, respectively 30,2%. And chromosome 11 was involved in 34 translocations or 22.82% of the total yield (Table 4).

Table 4 Frequencies of translocations involved chromosomes 1, 4 and 11.

Chromosome involved in	***AST(%)***	***ASD(%)***	***Insertions (%)***	***Complex exchanges (%)***
#1	52,35%	41,86%	43,75%	42,42%
#4	30,2%	37,21%	31,25%	36,36%
#11	22,82%	25,58%	25%	33,33%

Out of the 662 cells analyzed, 157 cells (23.72%) were found to be aberrant. Translocations (complete and incomplete) were found in 23.7% of the total cells (102 reciprocal and 55 non-reciprocal), with a genome frequency of 0.714 ± 0.033. Among 157 aberrant cells, 28 cells (or 17.83%) were associated with one or more complex exchanges (26 with one complex exchange, one cell with 2 complex exchanges, and one cell with 5 complex exchanges). We observed 14 cells (or 8.9%) with insertions; from these 12 cells had one insertion and two cells had two insertions.

4. Discussion

Fluorescent *in situ* hybridisation (FISH) with whole chromosome paints has greatly facilitated the identification of chromosome aberrations and is now widely used for accurate biological dosimetry in radiation accidents [11-13].

Four months post exposure, we still found very high frequencies of all kinds of radiation-induced chromosome aberrations. As expected of unstable aberrations, dicentrics decreased rapidly after exposure (0.96 ± 0.061 vs. 0.425 ± 0.042) [1]. This result correlates with other authors, who reported an average half-time disappearance of lymphocytes containing dicentrics and rings to be 130 days after an accident [14]. These data suggest that cells with dicentrics are eliminated from the circulating system with time. The patients were also treated with growth bone marrow factors because of severe leukopenia, which is also a reason for the decrease in the amount of cells with unstable aberrations.

It is interesting that the genome frequency of total dicentrics detected by FISH was lower than that detected by Giemsa staining (0.250 $\pm$ 0.02 per metaphase cell versus 0.425 ± 0.042 per metaphase cell). This result could be explained by the miscounting of some dicentrics, because pancentromeric probes were not used in order to be sure that every one was dicentric. In contrast, the frequency of translocations (0.714 ± 0.03 per metaphase cell) is close to the initial frequency of dicentrics (0.96 ± 0.061 per metaphase cell) found right after the accident, but lower [1]. Theoretically, the initial frequency of dicentrics should be similar to the frequency of translocations years after the accident, because of the mechanism of radiation-induced double-strand breaks [15]. The observed lower frequency of translocations, compared to initial frequency of dicentrics, observed in the patient was due to a very high initial dose irradiation and loss of cells with complex aberrations, as well as the threat of acute radiation syndrome (ARS).

We observed 57 incomplete translocations compared to the 92 complete translocations, or approximately 35% incomplete translocations. Conventional theory holds that these aberrations should occur in equal number during the first mitosis following radiation exposure, and this has been observed by a number of investigators [16-18]. However, many studies have observed a higher frequency of symmetrical translocations [19-22].

It has been reported that in the absence of a pancentromeric probe, some asymmetrical translocations can be misclassified as symmetrical [23], leading to an apparent excess of symmetrical (and a corresponding deficit of asymmetrical) translocations. This result is not surprising, as the equality between symmetrical and asymmetrical translocations is postulated only for the first post-irradiation mitosis. It is well known that unstable aberrations, such as asymmetrical translocations, disappear in successive cell generations [24, 25]. In our work, the predominance of complete symmetrical translocations in the patient who exposed to a high dose was most likely due to fact that the samples were collected approximately 4 months after the accident.

Conventional theory holds that radiation-induced stable aberrations, such as translocations, should persist over time after radiation because they do not cause cell death through division [26, 27]. Other studies report the temporal decline of translocations level over the time [23, 28]. This has been demonstrated with results for victims of the Goiania accident [23, 28]. The latter study reported that the translocation frequencies were two to three times lower, as the difference is higher at doses >1 Gy.

5. Conclusions

This is a pilot study for assessing *in vivo* cytogenetic abnormalities in a person from Bulgaria who was accidentally exposed to ionizing radiation. From this study, it is not possible to relate the high frequency of translocation with a long-term biological effect, but it is a well-known fact that ionizing radiation increases the risk of cancer in some individuals. The assessing of kinetics of persistence of translocations *in vivo* will be the subject of further study.

Author Contributions

All authors contributed equally.

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Competing Interests

The authors have declared that no competing interests exist.

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