Analysis of γ-rays induced chromosome aberrations: A fingerprint evaluation with a combination of pan-centromeric and pan-telomeric probes.

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Abstract.

Purpose: To evaluate the types of induced chromosome aberrations after the exposure of peripheral blood to γ-rays by the simultaneous detection of all centromeres and telomeres. To analyse the suitability of different radiation fingerprints for the assessment of radiation quality in cases of recent exposures.

Material and methods: Peripheral blood samples were irradiated at 2, 4 and 6 Gy of γ- rays. Cytogenetic analysis was carried out by fluorescence in situ hybridization (FISH) technique with pan-centromeric and peptide nucleic acid (PNA)-telomeric DNA probes. Cells were analysed using a Cytovision® FISH workstation, chromosome aberrations and the length of the acentric fragments were recorded.

Results: The total number of the incomplete chromosome elements was 276. The ratio between incomplete elements and multicentrics was 0.38. The number of acentrics was 1096, 71% were complete acentrics, 15% incomplete acentrics, and 14% interstitial fragments. The relative length of complete, incomplete and interstitial acentrics fragments were 2.70±0.04, 1.91±0.07, and 1.42±0.04 respectively. The mean value of the F-ratio was 11.5 higher than the one, 5.5, previously obtained for α-particles. For the G-ratio there was no difference between γ-rays and α-particles, 2,8 and 2,8 respectively. The mean value of the H-ratio for γ-rays, 0.25, was lower than for α-particles 0.40.

Conclusion: The results support that the percentage of incomplete chromosome aberrations depends on radiation type; low-linear energy transfer (LET) radiation would produces less incomplete aberrations than high-LET radiation. The F- and H-ratios seem to be good indicators of radiation quality, although a real estimation of the H-ratio is only possible using pan-telomeric probes.

Introduction

Fluorescence *in situ* hybridization (FISH) technique, using whole chromosome DNA probes, has improved the dose assessment of past radiation exposures by the analysis of translocations (Lucas et al. 1992, Natarajan et al. 1996, Lindholm et al. 2002, Edwards et al. 2005). Chromosome p*ainting* techniques, using two- or three-color FISH detection, have shown a large variety of complex chromosome aberrations involving three or more breaks in two or more chromosomes (Savage and Simpson 1994). The development of multifluor FISH (mFISH), in which all human chromosome pairs are differently painted (Speicher et al, 1996), has greatly improved the ability to detect complex aberrations (Anderson et al. 2003, Wu et al. 2003). The *painting* technique has also shown that in some induced exchanges, while one chromosomal end of one double strand break (DSB) joins a chromosomal end of another DSB, one or more broken ends seem to be unrejoined. However, most of these incomplete aberrations are in fact complete ones where the exchanged portions have lengths below the resolution of the painting technique (Kodama et al. 1997, Wu et al.1998, Fomina et al. 2000). The application of FISH using a peptide nucleic acid (PNA)-telomeric DNA probe (Lansdorp et al. 1996) allows an accurate detection of the 92 telomeres in human lymphocytes, and as a consequence allows one to identify true incomplete elements (Boei et al. 1998). Using FISH some authors have described a LET dependence in the percentage of true incomplete elements (Deng et al. 2000, Mestres et al.2004), whereas others did not find such dependence (Boei et al. 2001, Wu et al. 1999, Fomina et al. 2001).

It has been suggested that some ratios of specific radiation-induced chromosome aberrations can be used as fingerprints for radiation quality. This is due to the different pattern of energy

deposition between densely or sparsely radiation, and to the likelihood of two initial double-strand breaks to rejoin illegitimately (Brenner & Sachs 1994, Mitchell et al. 2004).

In solid stained metaphases several fingerprints of radiation quality have been proposed: inter-chromosomal to intra-chromosomal inter-arm aberrations, the F ratio, dicentrics to rings (Brenner & Sachs, 1994); inter-chromosomal to intra-chromosomal intra-arm aberrations, the H-ratio, dicentrics to interstitial deletions (Bauchinger & Schmid 1998); inter-arm to intra-arm intrachromosomal aberrations, the G ratio, centric rings to interstitial deletions (Sachs et al. 1997). In this sense, high-LET radiation like α-particles and neutrons induces more intrachromosomal, and intra-arm aberrations than low-LET radiation. All of these fingerprints are based on unstable aberrations and for this reason can not be used as retrospective biomarkers of radiation quality. The FISH techniques using two, three or 24 color detection, as well as the high resolution multicolor chromosome band FISH technique (mBAND) (Chudoba et al. 1999) have given the possibility to describe other fingerprints: F- and H- ratios, now considering translocations to pericentric or paracentric inversions respectively (Brenner et al. 2001); the S(I)-ratio of complete exchanges to incomplete rejoinings, and the S(II)-ratio of apparent complete exchanges to hidden complete exchanges, in this case combining whole-chromosome probes with pan-telomeric detection (Lucas 1998); the I-ratio, simple to complex aberrations, like translocations to inversions (Deng et al. 2000). Analyzing by mBAND Russian nuclear workers occupationally exposed from 1949 onward to plutonium, γ-rays, or both, it has been described that intrachromosomal aberrations were only significantly increased in the group of individuals exposed to plutonium but not in those exposed to γ-rays (Hande et al. 2003, Mitchell et al 2004). More recently and using mFISH technique Hande et al. (2005) indicated that complex transmissible translocations were significantly increased in the same

group of nuclear workers, suggesting that both, intrachromosomal type aberrations and complex transmissible translocations, could be a good permanent biomarkers of densely ionizing radiation exposure in retrospective analysis.

For recent exposures, the most accepted method for biodosimetry is the analysis of dicentrics alternatively plus rings observed in solid stained metaphases. Using this method the F-, G- and H- ratios can be achieved. As indicated above, G- and H-ratios consider the interstitial deletions. However, in solid stained metaphases terminal or interstitial deletions can not be clearly distinguished (International Atomic Energy Agency, IAEA, 1986), and only small deletions appearing as paired dots called "minutes", and larger acentric rings with a visible lumen, have been considered as interstitial deletions (Bauchinger & Schmid 1998). In a previous study from our laboratory the analysis of the acentric lengths induced by α-particles showed a great variability in the size of interstitial deletions (Mestres et al. 2004).

In the present study, pan-centromeric and PNA-telomeric probes have been used to estimate the percentage of true incomplete aberrations after γ-radiation exposure, and an analysis of the length spectra of the acentrics fragments is also presented.

Materials and methods

Irradiation conditions

Peripheral blood samples from a 37-year-old healthy male with no history of exposure to clastogenic agents, including radiation, were obtained by venipunture and collected in

heparinized tubes. Donor gave its informed consent and the proper rules were followed for obtaining human samples. The samples, maintained at 37 °C since 30 minutes before until 1h after irradiation, were irradiated at 2, 4 and 6 Gy, in a ^{60}Co source (Theraton-Phoenix) located at the "Hospital de la Santa Creu i Sant Pau (Barcelona)". Dose rate was 138, 37 cGy min^{-1}. Dose calibration was performed with a Farmer electrometer (Ber, UK), model 2570, with a 0.6 cm^3 ionizing chamber, model 2571. During irradiation the IAEA (1986) recommendations were followed.

Culture conditions and Fluorescence in situ *hybridization*

Peripheral blood was cultured for 48h in Roswell Park Memorial Institute (RPMI) 1640 medium (Biochrom, Cultek S.L., Madrid, Spain) supplemented with 20% foetal calf serum (Biochrom), antibiotics (Biochrom), phytohaemagglutinin (Biochrom) and 12 μg mL^{-1} of bromodeoxyuridine (Sigma-Aldrich, Barcelona, Spain). Colcemid (Biochrom) was added 2h before harvesting. The frequency of first-division metaphases, determined by the fluorescence plus Giemsa (FPG) technique in parallel slides, was higher than 95%. The simultaneous hybridization with the PNA pan-telomeric (Applied biosystems, Foster City, CA USA) and the pan-centromeric (Cambio, Dry Drayton, Cambridge, UK) probes was carried out as previously described (Mestres et al. 2004).

Microscope analysis and scoring criteria.

Only well spread metaphases containing the full set of 92 pairs of telomeric signals and 46 centromeric signals were considered. Once an abnormal cell was observed, each fluorochrome

image (Fluorescein isothiocyanate (FITC), Cyanine 3 (Cy3) and 4',6-Diamidino-2-phenylindole (DAPI)) was digitalised using a Cytovision® FISH workstation (Applied Imaging, Newcastle upon Tyne, UK). The chromosome aberrations analyzed were: multicentric chromosomes (dic-, tric- and tetracentrics), rings and acentrics fragments. The presence of telomeric signals at the ends of each piece was recorded. An apparently normal chromosome without telomeric signals at the end of one or both arms, was recorded as chr(+,-) or chr(-,-) respectively. Dicentrics lacking a telomeric signal at the end of one arm were recorded as dic(+,-). Acentric fragments were classified as ace(+,+) when the telomeric signals were present at both ends, and as ace(+,-) when the telomeric signals were not recognizable at one end of the acentric. In only a few small acentric fragments it was difficult to distinguish if there were just one telomeric signals in one end or if there were two superposed telomeric signals. In these cases, if the rest of the metaphase showed 90 telomeric signals, the acentric fragment was recorded as an ace (+,+). Circular acentric forms without telomeric signals and with a visible lumen were recorded as acentric rings (r_{ace}). Fragments lacking telomeric signals at both ends were recorded as ace (-,-). This category could include some acentric rings. The length of each acentric fragments was recorded after the description of all abnormalities observed. Taking into account the variability in chromosome size among metaphases, chromosome 1 was used as the reference. Assuming that the relative length (in percent of the total haploid chromosome length) of chromosome 1 is 8.3 (Stephens et al. 1990), the relative length for each recorded acentric fragment was calculated, multiplying the length of the fragment by a $8.3/L_1$ factor, where L_1 is the length of a chromosome 1 in the same metaphase.

Results

In the present study 1143 metaphases were analyzed, 551 of them with aberrations. The number of multicentrics (dic- , tri- , and tetracentrics) at 2, 4 and 6 Gy was 144, 373, and 201 respectively. At 2 and 4 Gy 142 dicentrics and 2 tricentrics, and 371 dicentrics and 2 tricentrics, and at 6 Gy the multicentrics observed were 190 dicentrics, 10 tricentrics and one tetracentric. Considering tricentrics and tetracentrics as two and three "dicentric equivalents", the number of dicentric equivalents was 734. As can be seen in table 1, the intercellular distribution of dicentric equivalents followed a Poisson, checked by the dispersion index (variance/mean) and the normalized unit of this index (Rao & Chakravarti 1956).

There are two main forms of incomplete elements (table 2), incomplete chromosome chr (+,-) and terminal acentric fragments ace (+,-). Considering chr (-,-) as two chr (+,-), and dic (+,-) as one chr (+,-), the total number of chr (+,-) was 117, and the total number of ace (+,-) was 159. So, the total number of incomplete elements was 276, 42% chr (+,-) and 58% ace (+,-). The relative percentage of these incomplete elements did not show dose dependence. The ratio between incomplete elements and multicentrics was 0.38. The number of acentric fragments was 1096; 71% of them complete ace (+,+); 15% incomplete ace (+,-) and 14% interstitial deletions ace (-,-). These proportions were constant at 2, 4 at 6 Gy.

In the present study 734 dicentric equivalents and 66 centrinc rings were observed. Thus from the total 1096 acentric fragments, 296 would be considered as extra acentrics in solid- stained metaphases. Alternatively if acentric rings are included, the number of extra acentrics increases up to 308.

The number of dicentrics was 142, 371 and 190 at 2, 4 and 6 Gy respectively, and the number of centric rings was 10, 38 and 18. The F-ratio (the ratio of dicentrics to centric rings) was 14.2, 9.8 and 10.6 at 2, 4 and 6 Gy respectively, and the mean F-ratio value-was 11.51. The number of interstitial deletions, including interstitial fragments and acentric rings was 34 at 2 Gy , 83 at 4 Gy and 53 at 6 Gy. The G-ratio, between interstitial acentrics and centric rings at 2, 4 at 6 Gy were 3.40, 2.18 and 2.94 respectively with a mean of 2.84. The H-ratio, between interstitial acentrics and dicentrics were 0.24, 0.22 and 0.28 respectively, with a mean of 0.25.

Table 3 shows the total number of acentric fragments grouped by their relative lengths from <0.5 to >8. Figure 1 shows the relative proportion of each acentric type. The mean length (±SE) of complete acentrics (2.70 ± 0.04) was higher than the one of incompletes (1.91 ± 0.07) and interstitial fragments (1.42 ± 0.04).

Discussion

In the present study, after γ-irradiation the ratio between incomplete elements and multicentrics was 0.38. The same ratio of 0.38 was observed after X-rays irradiation (Boei et al. 2000), although after α-particle irradiation a ratio of 1.00 was observed (Mestres et al. 2004). These results support the idea that the percentage of incomplete chromosome aberrations depends on radiation type. The complexity of the induced DNA damage (Goodhead et al. 1993) and the efficiency to be repaired (Jenner et al. 1993) seem to be different between low- and high-LET radiation. In this sense, and assuming that none of the chr (-,-) or ace (-,-) are rings, the total number of unrejoined break free ends was chr (+,-) + 2 chr (-,-) + dic(+,-) + ace (+,-) + 2 ace (-

,-) = 592: cryptic misrejoinings and or extra acentric rings would reduces this total. The total number of misrejoined free ends was at least 2[dic (+,+) + dic (+,-) + ace (+,+) + r + r_{ace}] = 3158. The ratio of unrejoined to misrejoined free ends is at most ~1/6, half than the one observed after α-particle irradiation ~1/3. Using whole-chromosome probes in combination with pan-telomeric probe to analyze the incomplete elements affecting specific chromosomes, differences in the percentage of incomplete elements for low and high LET radiation were described by Deng et al. (2000), but others did not find such differences (Fomina et al. 2001, Boei et al. 2001).

In the present study, after γ-irradiation, the relative percentage of the two incomplete forms was 42% of chr(+,-) and 58% of ace (+,-), different than those reported by Boei et al. (2000) after X-rays irradiation (60 and 40% respectively), and similar than those reported after α-particle irradiation (44 and 56%, respectively). In the same sense, from the 276 incomplete elements observed, and due to the presence of cells with multiple aberrations, it was possible to consider 108 pairs of incomplete elements: 57 (53%) pairs of chr (+,-) and ace (+,-); 8 (7%) pairs of chr (+,-) and chr (+,-); and 43 (39%) pairs of ace (+,-) and ace (+,-). Boei et al. (2000) reported that the pairs of incomplete chromosomes [chr (+,-),chr (+,-)] were four times more frequent than the pairs of incomplete acentrics [ace (+,-), ace (+,-)], and suggested that a break free end of a centromere-containing chromosome fragment was more prone to remain unrepaired. However, after α-particle irradiation the pairs of incomplete chromosomes were two times less frequent than the pairs of incomplete acentrics, and it was argued that the likelihood to repair a centromere-containing or a non-centromere-containing fragment would depend on the type of radiation Mestres et al. (2004). In the present study the number of incomplete chromosome pairs is five times lower than incomplete acentric pairs. As conclusion, the results indicate that

the likelihood to repair a centromere- or a non centromere- incomplete element, does not depend on radiation quality neither on the presence of a centromere.

In the present study, where the methodology used allows to distinguish unequivocally multicentrics, centric rings, and interstitial and terminal deletions, the mean value of the ratio between dicentrics and rings (F-ratio) was 11.51 ± 2.37. This value is in agreement with those reported in the literature for low-LET radiation ~15 (Lloyd et al. 1986, Hlatky et al. 1992, Brenner & Sachs 1994), and clearly higher than the ones described for high-LET radiation ~6 (Brenner and Sachs, 1994, Mestres et al. 2004). These results support the suitability of using the F-ratio as an indicator of radiation quality for recent exposures. Nevertheless a LET dependence in the ratio between dicentrics and rings was not observed by several authors (Bauchinger & Schmid 1997, 1998, Lucas et al. 1999, Kodama et al. 1999). Sasaki et al. (1998) indicated that there is a general trend of the F value to be lower for high-LET than for low-LET, and that this difference is more pronounced at low doses and diminished with increasing dose. Wu et al. (2001) compared the F-ratios obtained by premature chromosome condensation technique (PCC), that condenses chromosomes mostly in G_2 phase of the cell cycle, and conventional colcemid mitotic arrest, analyzing metaphases. These authors observed that heavily damaged cells which were less likely to reach mitosis or reach it at a later time, were responsible for the differences in the F ratios generated from interphase (PCC) and metaphase analysis after to high-LET radiation. However, in the same study a lack of differences between high- and low-LET radiation was observed in the F-ratio generated in the metaphase analysis.

Bauchinger & Schmid (1998) suggested that instead of ratios between inter- and intrachromosomal aberrations (F ratio), ratios between intra-arm to inter-arm chromosome

intrachanges (G ratio) or ratios between intra-arm intrachanges to interchanges (H ratio) would have more potential to be used as indicators of different LET. In the present study, the mean value of the G-ratio was 2.84 ± 0.61 close to the one observed by Deng et al. (2000) ~2.0 and to the one calculated by the random walk model ~2.5 (Wu et al. 1997). However, there was no difference between the G-ratio from the present study and the one after α-particle exposures ~2.5 (Mestres et al. 2004). The lack of differences in the G-ratio between different radiation qualities was also described by Deng et al. (2000). For the H-ratio, the mean value of the present study 0.25 ± 0.03 is lower to the one after α-particle exposure ~0.4 (Mestres et al. 2004). This result agrees with those by Deng et al. (2000), where the H-ratio was lower for γ-rays (~0.3) than for neutrons (~0.6).

The G- and H- ratios observed in the present study are different to the ones reported by Bauchinger & Schmid (1998), ~0.9 and ~0.1 respectively. In that study Giemsa-stained metaphases were analyzed, and interstitial deletions included large acentric rings and double minutes, the last ones considered when the width of the acentric was smaller than the diameter of a chromatid. With this criterion only small acentrics are considered as interstitial ones. In the present study interstitial deletions were unequivocally distinguished, and from the 91 smallest acentric fragments, with relative sizes between 0 and 1, only a 38% were ace (-,-). In agreement with Mestres et al. (2004), this result support that by the analyses of Giemsa-stained metaphases, to consider only the smallest acentrics as interstitial deletions leads to unrealistic values, because the size distribution of the real interstitial fragments, ace (-,-), is not different from the terminal deletions ace (+,-), and the majority of the smallest fragments are not interstitial ones.

In conclusion, the percentage of incomplete chromosome aberrations depends on radiation type, low-LET produces less incomplete aberrations than high-LET radiation, and from three fingerprints evaluated, it can be concluded that the F- and H-ratios have more potential to be used as indicators of radiation quality. However, a realistic estimation of the H-ratio is only possible using a pan-telomeric probe to distinguish clearly the interstitial deletions.

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Table 1. Cell distribution of dicentric equivalents

Dose (Gy)	Total Cells	Cells with aberrations	Cells with x dicentrics							dic	y	±SE	DI	*u*
			0	1	2	3	4	5	6					
2	643	153	515	114	10	4				146	0,23	±0,02	1,08	1,37
4	400	302	143	165	71	17	3	1		375	0,94	±0,05	0,86	-1,91
6	100	96	7	32	25	20	10	5	1	213	2.13	±0,13	2,58	-1,06

Dose in Gray (Gy); dic, total dicentric equivalents observed; y ± SE, frequency per cell of dicentric equivalents ± standard error [SD/(N)½]; DI, dispersion index variance/mean; *u* values between ±1.96 indicate a Poisson distribution.

Table 2. Cytogenetic results after γ-irradiation, using pan-centromeric and pan- telomeric probes.

Dose (Gray)	chr (+,-)	chr (-,-)	dic (+,+)	dic (+,-)	r	ace (+,+)	ace (+,-)	ace (-,-)	r_{ace}
2	16	3	146	0	10	153	28	32	2
4	48	8	372	3	38	396	101	76	7
6	22	2	211	2	18	230	30	50	3
Total	*86*	*13*	*729*	*5*	*66*	*779*	*159*	*158*	*12*

chr (+,-) and chr (-,-), chromosomes lacking one or two telomeric signals; dic (+,+) or dic (+,-), dicentric equivalents (including appropriate contributions from tricentics and tetracentrics) with telomeric signals in the two or one extremes respectively; (r), centric rings; ace (+,+), ace (+,-), ace(-,-), acentrics with two, one, or without telomeric signal; (r_{ace}), acentric rings.

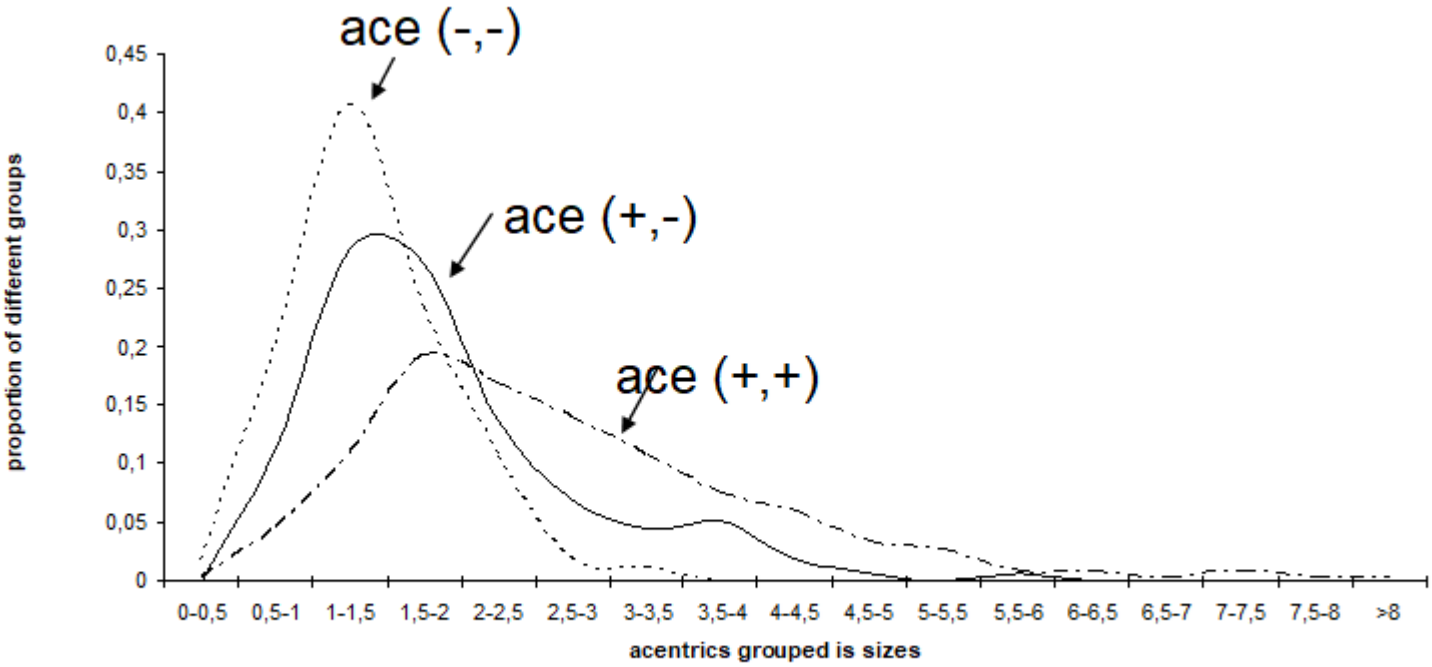
Table 3. Distribution of acentrics by their relative length.

Relative length	ace (+,+)	ace(+,-)	ace(-,-)
0-0,5	2	0	3
0,5-1	36	18	32
1-1,5	85	45	64
1,5-2	150	43	37
2-2,5	132	22	17
2,5-3	109	11	3
3-3,5	84	7	2
3,5-4	58	8	0
4-4,5	47	3	0
4,5-5	27	1	0
5-5,5	21	0	0
5,5-6	7	1	0
6-6,5	7	0	0
6,5-7	3	0	0
7-7,5	7	0	0
7,5-8	2	0	0
>8	2	0	0

The relative length for each recorded acentric fragment was calculated, multiplying the length of the fragment by ($8,3/L_1$). Where L_1 is the length of chromosome 1 in the same metaphase, and 8,3 is the relative length of chromosome number one (Stephen et al. 1990)

Figure captions

Figure1. Distribution of each type of acentrics according to their relative length.



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