

Experimental radiobiology

Genetic determination of chromosomal radiosensitivities in G0- and G2-phase human lymphocytes

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Abstract

Background and purpose: The radiosensitivity of human lymphocytes measured using a G0- or G2-assay has been linked with an individual's risk of developing normal tissue complications following radiotherapy. This study was performed to increase basic knowledge of the genetics of the human radiation response, and chromosomal aberration induction in particular.

Materials and methods: The study was carried out with blood samples taken from 15 monozygotic twin pairs. G0-assay was performed for cells irradiated with 6 Gy counting only deletions and G2-assay for cells irradiated with 0.5 Gy scoring only chromatid breaks.

Results: The mean number of deletions measured at 6 Gy for all 30 samples using the G0-assay amounted to 2.96 ± 0.37 (means \pm SD), which corresponds to a coefficient of variation (CV) of 13%. There is a highly significant intra-pair correlation for this number among twins ($r^2 = 0.911$) demonstrating that this parameter is mostly determined by genetic factors. According to the mean number of deletions, a theoretical classification based on the definition $\leq MV - SD$ as resistant, $MV \pm SD$ as normal and $\geq MV + SD$ as sensitive was made, identifying two pairs as sensitive or resistant, respectively, while nine were normal and two pairs are intermediate. For chromatid breaks measured at 0.5 Gy with the G2-assay the mean number was 1.35 ± 0.42 (means \pm SD) corresponding to a CV of 31%. There was again a strong intra-pair correlation among twins with $r^2 = 0.837$ showing that this sensitivity is also determined mostly by genetic factors. There was, however, no inter-assay correlation between the G0- and G2-sensitivity ($r^2 = 0.006$) demonstrating that these two sensitivities depend on different genetic factors.

Conclusion: The chromosomal radiosensitivity of lymphocytes as defined by G0- or G2-assay is largely determined by different genetic factors, which may allow the use of genetic profiling as an indicator of the respective individual radiosensitivity.

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Radiotherapy has become a very efficient tool to control tumours at many different sites. However, quite often the maximum dose applicable is limited by the occurrence of late normal tissue effects [8]. These effects were found to show a substantial variation both in the extent as well as in the kinetics even after identical treatment protocols [11,15,43,46–48]. That is while some patients show extensive side effects even shortly after radiotherapy, others are free of these effects even after a prolonged follow-up.

It was recently shown for late effects that the overall risk could be described by a probability, p_a , to develop a certain effect within a year [27]. This probability was found to remain constant after treatment in the range of 1–5% per year

[27]. Due to the substantial variation there is a great interest to identify parameters allowing the prediction of the individual risk of late effects. So far numerous parameters were studied such as the surviving fraction at 2 Gy, the number of initial or remaining single- or double-strand breaks (dsbs), as well as the amount of various types of chromosomal damage for review see [14,24,36]. Recently also genetic markers such as gene expression profiles as well as single nucleotide polymorphisms (SNPs) were analysed for their potential to predict the individual risk for review see [2,9]. Twin studies have revealed a marked heritable component of the transcriptional response to radiation exposure in that the post-radiation variation in the expression level of

several genes was significantly greater ($P < 0.001$) among twin pairs than within twin pairs [19].

It was previously shown by us for 86 breast cancer patients, that the radiosensitivity of lymphocytes as determined by the G0-assay can be used to predict the risk of late effects [26]. Blood samples were taken from patients and were irradiated in vitro with 6 Gy. Forty-four hours later lethal chromosome aberrations were measured using the G0-assay. Patients, who were termed to be sensitive, because the number of lethal aberrations was high, showed on average a more than twofold higher annual risk, p_a , to develop skin fibrosis than patients with an intermediate or low number of aberrations. Comparable observations were made by West et al. [49] using the colony forming assay for patients with cervical and de Ruycck et al. [20] analysing chromosomal damage of the G2-phase for patients with endometrial cancer.

The first evidence of a heritability of cellular radiosensitivity was shown by Roberts et al. [40] followed by a recent study by Wu et al. [50] both providing that the G2-radiosensitivity measured for lymphocytes might be strongly influenced by genetic factors. The study of Wu et al. [50] was performed with blood samples taken from 148 pairs of monozygotic twins and the G2-sensitivity was measured at 1.25 Gy. Within twin pairs this sensitivity showed a significant correlation with $r = 0.55$ and it was calculated that the sensitivity is determined at least to 62.5% by genetic factors [50]. A similar observation was previously made by Cloos et al. [17] for the bleomycin sensitivity, which is known to correlate with G2-radiosensitivity [1]. These findings, however, do not necessarily imply that such a genetic determination also applies for the radiosensitivity as determined with the G0-assay, since these two sensitivities – G2- and G0-radiosensitivity – were shown to be not correlated with each other [3,44]. As mentioned by Baeyens et al. [3] this lack of correlation “points to the fact that different DNA damage processing mechanisms are operating in G0- and G2-phase of the cell cycle”. Both, chromosomal damage detected either by G0- or G2-assay, are considered to result from non- or mis-rejoined dsbs. This type of DNA lesion is known to be repaired by the two processes ‘non-homologous end-joining’ (NHEJ) and ‘homologous recombination’ (HR) which differ in cell cycle dependence. While NHEJ was shown to be active in all cell cycle phases, HR is involved only in late S- and G2-phase [37,41].

It was the aim of this study to test, if and to which extent the radiosensitivity as detected by the G0-assay is also determined by genetic factors and whether these can be correlated with modulators of the G2-radiosensitivity. This study was carried out with blood samples taken from 15 monozygotic twin pairs and radiosensitivity of lymphocytes was determined with both G0- and G2-assay.

Materials and methods

Recruitment of twins

Fifteen monozygotic twin pairs were recruited by Health-Twist GmbH (Berlin) using the Berlin Twin Register [10]. Medical history and physical examination prior to study entry was conducted by HealthTwist. Zygosity was determined

using five microsatellite markers coamplified by polymerase chain reaction [7]. The probability of a dizygotic twin pair to share all marker alleles by chance is 0.006. The overall rate of correct classification is >99%. Twins were all male with an age ranging between 18 and 64 and a mean value of 32 years. Heparinised peripheral blood samples (10 ml) were collected and were analysed in the laboratory at least 24 h later. All donors had given written informed consent and the study design was approved by the local Ethical Committee.

Irradiation

Irradiation was performed at room temperature using a Seifert X-ray machine operating at 220 kVp, 15 mA with 0.5 mm Cu filter at a dose-rate of 2 Gy/min.

G0-assay

G0-assay was carried out as described previously [26]. Blood samples of 2 ml were split into four fractions of 0.5 ml, with two being irradiated with 6 Gy and two for control. Immediately after irradiation blood was diluted in 4.5 ml RPMI (Gibco) medium supplemented with 15% FCS and stimulated to enter cell cycle with 2.5% PHA (Boehringer) followed by an incubation at 37 °C for 6 h. Lymphocytes were blocked in metaphase by the addition of 0.2 µg/ml Colcemid (Gibco) for 4 h. Thereafter, cells were treated in hypotonic KCl-solution (0.075 M, Sigma) and fixed several times in Carnoy’s fixative. Fixed cells were dropped on pre-cleaned wet slides, stained with 2% Giemsa (Sigma) for 7 min and embedded permanently with Entellan (Merck).

Analysis of metaphase fragments was performed by means of light-microscopy. Routinely, three to four encoded slides with 25–50 cells per slide were scored using a Zeiss microscope equipped with a cooled CCD camera (CFI/1 FMCC, Kappa-Messtechnik, Germany) and an image analysis system. Metaphase spreads were screened for deletions, which is the number of fragments scored in irradiated cells corrected by the number of 46 chromosomes as seen for non-irradiated cells. These deletions are considered to result from terminal and interstitial deletions [18].

G2-assay

G2-assay was carried out as previously described by Scott et al. [44]. Briefly, blood samples were split into two fractions of 1 ml in 25 cm² culture flasks (Cellstar), which were diluted 1:10 with RPMI media (Gibco) containing 15% FCS and 25 µg/ml Penicillin/Streptomycin (Gibco). T-lymphocytes were immediately stimulated to enter cell cycle by the addition of 2.5% PHA (Boehringer) followed by an incubation at 37 °C for 72 h. One fraction was then irradiated with 0.5 Gy followed by a further incubation at 37 °C for 30 min. Colcemid was added and cells were harvested after 60 min. Further preparation of metaphase arrested cells was identical as described for the G0-assay. The metaphase cells were scored for fragments, isochromatid fragments, triradials and translocations, which were summarised as chromatid breaks.

Statistics

For each sample at least 80 metaphase cells were scored using coded slides. The values presented are mean values

with standard errors of the mean (SEM). Normal Distribution of phenotypic values was tested by Kolmogorov–Smirnov tests. To test the hypothesis of a genetic component of radiosensitivity, within-pair Pearson correlation coefficients were calculated. A p value of 0.05 was considered significant.

Results

G0-assay

Fig. 1A shows the variation of G0-radiosensitivity as measured for all 30 twins. Experiments were carried out on single blood sample per twin without any replicates and assayed on the same day blind for the twin status. Blood samples were split for G0- or G2-assay. For the G0-assay samples were irradiated with 6 Gy and the number of deletions was measured 72 h after irradiation. The number of deletions was found not to be correlated with the age of patient ($r^2 = 0.0001$). The variation in G0-sensitivity observed was described by a normal distribution with a mean value of $MV = 2.97$ deletions per cell and a standard deviation of $SD = 0.37$. The corresponding coefficient of variation amounts to $CV = 13\%$.

Twins were classified as resistant ($\leq MV - SD$), normal ($MV \pm SD$) or sensitive ($\geq MV + SD$) as previously defined [24]. According to these criteria, two of the 15 twin pairs were classified as resistant (see Fig. 1, open bars and circles), two as sensitive (Fig. 1, black bars and circles) and nine as normal (see Fig. 1, grey circles and squares). Only for two pairs the classification was ambiguous, because for both pairs the G0-radiosensitivity was just at the border between these classes with one twin being either resistant

or sensitive and the other being normal (Fig. 1B, grey diamonds).

For G0-sensitivity there was an excellent correlation among the twins with $r^2 = 0.911$ ($p < 0.001$) (Fig. 1B). That is when G0-sensitivity was high for one twin, this was also true for the other and *vice versa*. This result demonstrates that the radiosensitivity as determined with G0-assay is mostly determined by genetic factors.

G2-assay

Fig. 2A shows the variation of G2-sensitivity as measured for all 30 twins. Experiments were carried out on single blood sample per twin without any replicates and assayed on the same day blind for the twin status. The G2-assay was done at the same day and from the same blood sample as the G0-assay. For the G2-assay the blood samples were stimulated and 72 h thereafter cells were irradiated with 0.5 Gy and the number of chromatid breaks was determined. Again there was no correlation with the age of the twin ($r^2 = 0.013$). When data were analysed using a normal distribution, the mean value was calculated to be 1.35 chromatid breaks with a standard deviation of 0.42 corresponding to a CV of 31%. Within twin pairs there was again a highly significant correlation for the G2-sensitivity ($r^2 = 0.837$) (Fig. 2B), illustrating that the G2-radiosensitivity also depends mostly on genetic factors.

The theoretical classification in resistant ($\leq MV - SD$), normal ($MV \pm SD$) or sensitive ($\geq MV + SD$) resulted again in two resistant (Fig. 2B, open squares), eight normal (Fig. 2B, grey squares) and two sensitive (Fig. 2B, black squares) twin pairs. For three pairs (Fig. 2B, grey diamonds) the classification was intermediate, because the sensitivity was just at the border between two classes with one twin

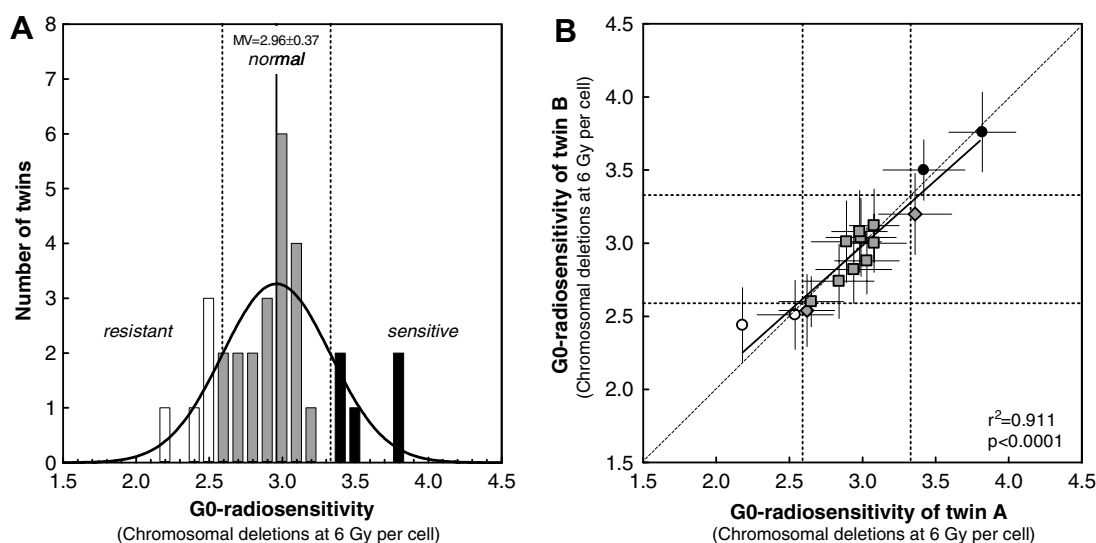


Fig. 1. G0-radiosensitivity of 15 monozygotic twin pairs. (A) Variation of radiosensitivity as measured with G0-assay. Immediately after irradiation with 6 Gy blood samples taken from twins were stimulated by 0.25% PHA followed by an incubation for 68 h, before cells were collected in metaphase with colcemid for 4 h. At least 80 metaphase cells per dose point were used to count the number of deletions per lymphocyte expressed as $MV \pm SD$. (B) Correlation of G0-radiosensitivity within monozygotic twin pairs. Values were taken from (A). Patients are defined as resistant (open bars/circles), normal (grey squares/ bars), sensitive (black bars/ circles) or intermediate (filled diamonds), dotted lines indicate SD; for details see text. Data were either fitted by normal distribution (A) or linear regression analysis (B). Error bars represent standard error of the mean.

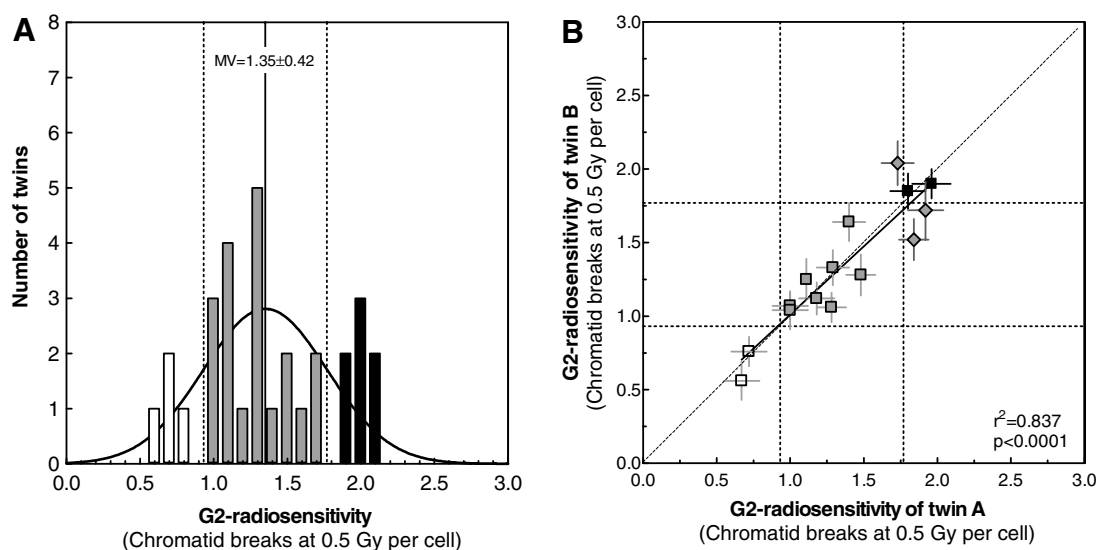


Fig. 2. G2-Radiosensitivity of 15 monozygotic twin pairs. (A) Variation of radiosensitivity as measured with G2-assay. Blood samples were first stimulated with 0.25% PHA followed by an incubation for 72 h. Lymphocytes were then irradiated with 0.5 Gy followed by an incubation for 30 min before cells were collected in metaphase with colcemid for 1 h. Metaphase cells were used to determine the total number of chromatid breaks per lymphocyte. (B) Correlation of G2-radiosensitivity within monozygotic twin pairs. Values were taken from (A). Patients are defined as resistant (open bars/circles), normal (grey squares/ bars), sensitive (black bars/ circles) or intermediate (grey diamonds), dotted lines indicate SD; for details see text. Data were either fitted by normal distribution (A) or linear regression analysis (B). Error bars represent standard error of the mean.

being sensitive and the other being normal (Fig. 1B, grey diamonds).

There was, however, no inter-assay correlation between the two sensitivities tested for all 30 samples as can be seen from Fig. 3. The correlation coefficient of $r^2 = 0.0055$ suggested that these two endpoints depend on processes, which are independent from each other.

Discussion

The aim of this study was to confirm the assumption that the individual radiosensitivity as determined with lymphocytes is mostly determined by genetic factors. The study was performed with blood samples taken from 15 monozygotic twin pairs. All twins were male and were checked for state of zygosity by using five microsatellites [7].

G0-radiosensitivity

For measurement of G0-radiosensitivity blood samples were irradiated *in vitro* with 6 Gy and lymphocytes were analysed for deletions 72 h thereafter. When compared to previous studies [12,26] the time interval between irradiation and fixation in metaphase was prolonged from 46 to 72 h. This prolongation was found to be necessary in order to achieve a sufficiently high number of metaphase cells, when blood samples were not taken directly in the lab but were delivered and analysed 20–30 h later (data not shown). From all types of chromosomal aberrations present only deletions were scored. This type of aberration was chosen, because it is the most frequent type of all aberrations induced and, therefore, its number appears to be a more robust indicator of the G0-sensitivity than the number of

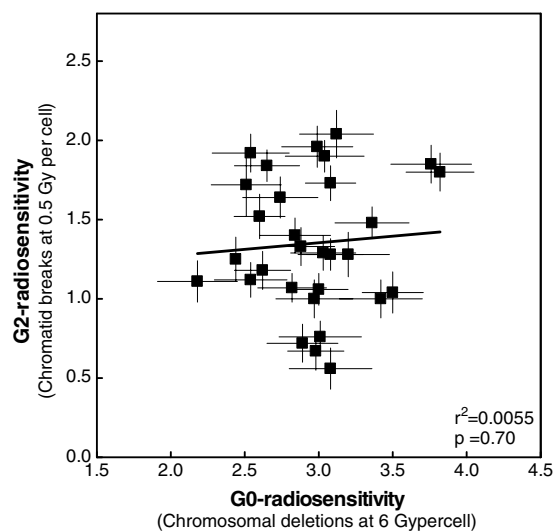


Fig. 3. No inter-assay correlation between G0- and G2-radiosensitivity for 15 monozygotic twin pairs. Data for G0- and G2-sensitivities are taken from Figs. 1 and 2A, respectively. Data were fitted by linear regression analysis. Error bars represent standard error of the mean.

dicentrics [12,26]. A similar result was reported by Rave-Fränk et al. [38].

For all 30 twins the mean number of deletions was determined to be 2.96 ± 0.37 per lymphocyte. This number was almost identical to the mean value of 3.02 ± 0.33 measured for a group of 30 healthy donors (data not shown). This result indicates that in respect to G0-radiosensitivity the twins of this study can be regarded as representative for healthy donors. The variation in G0-sensitivity was found to be

characterised by a CV of 13% (Fig. 1B). This value is fairly similar to those found in other reports, when radiosensitivity was determined even with different techniques [4,26,32,35,42].

Overall, these data clearly demonstrate that the G0-radiosensitivity measured for lymphocytes is mostly determined by genetic factors. It is expected that these factors will also control the G0-sensitivity of other cell types. Therefore, for a specific patient the sensitivity of lymphocytes can be used as a general indicator of the G0-radiosensitivity as already done in numerous studies [for review see 24].

So far the processes and with that the genes, which are responsible for the variation in G0-radiosensitivity, are not known yet [25]. It was shown that the variation in radiosensitivity as measured for several rodent cell lines as well as primary human fibroblasts using the colony assay mostly results from differences in dsb repair capacity [21–23]. Already small differences in this capacity of only 2–5% were found to be associated with huge differences in cell killing [30]. There are already numerous genes known to be involved in dsb repair. However, this does not necessarily imply that these genes are also responsible for the variation in dsb repair capacity as detected for human fibroblasts [25]. For instance, for the DNA PK complex, which is the central unit of NHEJ, a reduction in activity by only 20% was found to result in a clear decline of dsb repair capacity with a substantial increase in cellular radiosensitivity [28]. However, in twelve different human fibroblast strains with clear differences in dsb repair capacity, the DNA PK activity was characterised by only a modest variation, which did not correlate with the respective dsb repair capacity [29,31]. Probably variation in dsb repair capacity is caused by changes in other factors such as the chromatin structure [13,25], repair factors downstream of DNA-PK or only moderate protein modifications caused by SNP's [2].

G2-radiosensitivity

For G2-radiosensitivity, which was determined at 0.5 Gy, variation is described by a CV of 31% with a mean value of 1.35 chromatid breaks per lymphocyte (Fig. 2A). This variation, which is similar to that found in other reports [3,5,6,33,39], is clearly higher than that calculated for G0-sensitivity (see above). It is suggested that this difference results from the fact that for G2-assay the number of events counted is almost twofold lower when compared to the G0-assay (MV = 1.35 vs. MV = 2.96) and, therefore, the effect of statistical scatter might be higher. For the G2-assay only chromatid breaks were scored as done in most other studies [3,5,6,33,39,44]. The data obtained were fitted to a unipolar normal distribution as also observed by others [3,5,16,33,39]. Only in a very large study with 105 normal female donors performed by Scott et al. [44] there was an indication for a second population of about 10% with a higher level of chromatid breaks.

For the G2-radiosensitivity there was an excellent correlation among twins with a correlation coefficient of $r^2 = 0.837$ (Fig. 2B). A similar result was observed by Wu et al. [50] studying G2-sensitivity for 148 monozygotic twin

pairs with $r^2 = 0.303$. The lower correlation coefficient found in their study might result from the fourfold lower number of aberrations scored 4 h after irradiation. But other methodological differences may have contributed to the differing results as well.

The mechanisms controlling G2-radiosensitivity are not yet fully understood. It is generally suggested that this sensitivity is determined by HR, since this process is known to be involved in dsb repair, when cells are in the G2-phase [3,34,37,41]. However, data from Terzoudi et al. [45] indicate that the number of chromatid breaks detected by G2-assay also depends on the level of the cdk1/cyclin B activity during G2/M transition.

For the 30 twins analysed, there was no correlation between the G0- and G2-radiosensitivity (Fig. 3). Similar findings were previously reported by Scott et al. [44] and Baeyens et al. [3]. It is suggested that these two sensitivities are regulated by different mechanisms as outlined above. Despite this, both parameters were found to be useful to identify patients with an increased or decreased risk in late effects after radiotherapy [20,26]. Since these two sensitivities appeared to be independent of each other as discussed above, a patient showing both, an enhanced G0 as well an enhanced G2-radiosensitivity, may be characterised by an extraordinarily high risk for late effects. It will be interesting to test this hypothesis in our current study with breast cancer patients.

In summary, it is shown in this study with lymphocytes from 15 monozygotic twin pairs that both the G0- and the G2-radiosensitivity are largely determined by genetic factors, which, however, are not correlated with each other. The use of chromosomal aberrations as an indicator of the individual radiosensitivity might also help to determine genes that are relevant for this parameter by comparing the gene expression profile of "sensitive" with that of "resistant" twin pairs. This strategy is presently used to establish a profile that can be applied to identify patients with an enhanced individual radiosensitivity, which is suggested to be linked with an elevated risk for severe side effects after radiotherapy.

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