G2 chromosomal radiosensitivity and background frequency of sister chromatid exchanges of peripheral blood lymphocytes of breast cancer patients

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Background: Chromosomal alterations play an important role in carcinogenesis. Enhanced chromosomal radiosensitivity is shown for many cancer predisposition conditions including breast cancer. In this study chromosomal radiosensitivity and the frequency of background sister chromatid exchanges (SCE) in lymphocytes of normal individuals and breast cancer patients was compared.

Materials and Methods: G2 assay was performed on peripheral blood lymphocytes obtained from 60 breast cancer patients and 50 normal control. Blood culture was initiated and cells were irradiated with 1 Gy gamma-rays 4 h prior to harvesting. After metaphase preparations and slide making, chromatid aberrations were scored. For SCE studies, blood samples from 30 breast cancer patients and 30 normal control were studied. 24 hours after culture initiation, 5-bromodeoxy uridine (BrdU) was added and cells were harvested 48 hours after addition of BrdU. Slides were stained in Hoechst 33258 and exposed to UVA source, then stained in Giemsa.

Results: Results indicated that the frequency of radiation induced chromatid breaks was significantly higher in breast cancer patients compared to normal control (p<0.01). From radiosensitivity point of view, 12% of normal control and 47% of breast cancer patients showed elevated chromatid radiosensitivity. Frequency of background SCE was significantly higher in lymphocytes of breast cancer patients compared to lymphocytes of control (p<0.05). Conclusion: Elevated chromosomal radiosensitivity and higher frequency of SCE in lymphocytes of breast cancer patients might be indicative of genomic instability of these cells. Increased radiosensitivity could also be due to defects in DNA repair genes involved in breast cancer formation.

Keywords: Radiosensitivity, chromatid breaks, SCE, breast cancer patients, lymphocytes.

INTRODUCTION

Breast cancer is one of the most common cancers among females worldwide with an increasing trend even in countries with a low incidence rate (1, 2). In Iran the incidence of the disease is rising and affected people are relatively younger compared to their western counterparts (3, 4). During last four decades, increasing its incidence rates has made breast cancer one of the most frequent malignancies among Iranian woman (5). The association of chromosomal aberrations and rearrangements and tumor formation has been reported previously (6). It was shown that chromosomal translocations are involved in some human malignancies (7, 8) and that chromosomal aberrations (CA) are increased prior to clinical manifestation of cancer (9, 10). Chromosomal aberrations are manifestation of DNA damages induced directly or indirectly by various types of clastogenic chemical or physical agents. Both impaired DNA repair and genome instability are considered as factors underlying increased susceptibility to malignancy (11, 12). The biological importance of genomic instability and DNA repair mechanisms in cancer development are particularly well

illustrated by several heritable genetic disorders known as chromosome breakage syndromes, such as ataxia–telangiectasia (AT), Nijmegen breakage syndrome, Bloom syndrome, and Fanconi anemia, characterized by inherited chromosomal instability, radiosensitivity and cancer predisposition (12-14). Apart from these rare syndromes, the deficient DNA repair capacity has been proposed to be a predisposing factor in familial and sporadic breast cancer cases (15, 16). Genomic instability has also been described for various hereditary cancers including hereditary breast cancer (17, 18). About 10% of apparently normal individuals and 40% of breast cancer cases show elevated radiosensitivity i.e. in the range of AT heterozygotes, linking high radiosensitivity with predisposition to cancer (19). Radiosensitivity has been studied in breast cancer patients in terms of G2, micronuclei or comet assay (16, 19, 20-23). Despite current information, a consensus has not been reached on cellular assays and breast cancer risk. Reported data in the literature are contradictory. Some reported high radiosensitivity and reduced capacity of DNA damage repair of peripheral blood lymphocytes of breast cancer patients when exposed to ionizing radiation in terms of chromosomal aberrations (15, 24, 25), micronuclei (17, 18, 20, 23, 26, 27), and DNA fragmentation (16, 21, 22, 28, 29); while others reported no significant difference between the DNA repair capacity and formation of chromosomal aberrations (17, 22, 50). However, the relatively low number of papers published with the G2 or MN assay are not suitable for meta analysis and call for larger scale studies in different populations to rule out the usefulness of these methods for screening purposes and further understanding of occurrence of genome instability in breast cancer patients (27). There are also reports showing different yield of sister chromatid exchange (SCE) in lymphocytes of normal control and breast cancer patients. However because of limited studies, hence limited information, there is not a consensus agreement for using SCE as biomarker for screening patients with predisposition to breast cancer (31, 32, 33). Therefore, the development of assays with the potential of identifying cancer prone individuals is of prime interest in the field of cancer research.

The aim of this study was therefore to assess the radiosensitivity of lymphocytes of breast cancer patients in terms of radiation induced chromatid aberrations and background frequency of sister chromatid exchanges compared to lymphocytes of not affected control individuals in Iranian population.

MATERIALS AND METHODS

Patients and study subjects
A total of 60 breast cancer patients age ranging between 25 and 76 years (mean age 46 ± 13.9), and 50 (40 women and 10 men) normal (control) age ranging between 23 and 66 years (mean age 37.4 ± 11) were studied for G2 assay and radiosensitivity. For Sister chromatid exchange (SCE) studies 30 breast cancer patients were randomly selected among patients with the age range of 26-63 (mean age (46 ± 11) and 30 (20 women and 10 men) normal (control) age ranging between 27 and 65 years (mean age 37 ± 9). The study was approved by the Ethical Committee of the Faculty of Medical Sciences of the Tarbiat Modares University. All donors gave their informed written consent and completed a written questionnaire to obtain information related to their lifestyle, such as dietary habits, medical history and exposure to chemical and physical agents. Therefore, to limit confounding factors and be sure of the effects seen on samples is due to ionizing radiation, all samples had been screened to exclude previous radiation exposure, smoker, antibiotic consumption and viral infection at least one month prior to sample collection.

Whole blood culture and irradiation for radiosensitivity study
Venous blood samples were collected in
Cytogenetic radiosensitivity and SCE in breast cancer patients

Each blood sample was divided into two parts one as non-exposed and the other for exposure to gamma irradiation. Whole blood culture was prepared by adding 0.4 mL blood to 4.5 mL RPMI-1640 medium (Gibco BRL) supplemented with 0.2 mM L-glutamine, 15% fetal bovine serum (Gibco BRL), antibiotics (penicillin 100 IU/mL and streptomycin 100 µg/mL). Phytohemaglutinin (PHA) at a final concentration of 5 µg/mL was added to the culture medium to stimulate division of lymphocytes. Blood cultures were incubated at 37 °C for 72 hours. In irradiated group, four hours before harvesting culture vessels were exposed to 1 Gy gamma rays generated from a 60Co source (Theratron 780C, AECL, Kanata, Canada) at source to sample distance of 80 cm with a dose rate of 165.8 - 156.9 cGy/min at room temperature (23 ± 2 °C). Colcemid (Sigma Aldrich) at a final concentration of 0.1 µg/mL was added 2 h prior to harvesting. The cells were then collected with centrifugation (1200 rpm, 10 min) and pellets were resuspended in hypotonic solution (0.075 M KCl, Merck) for 10 min at 37 °C. Cells were centrifuged and hypotonic solution was removed by aspiration. Cells were then fixed three times in methanol: glacial acetic acid (3:1) before spreading on wet slides. Slides were air dried and stained in 4% Giemsa for 10 min. Chromatid breaks were scored and analysed in well spread metaphase cells under x100 oil immersion light microscope (Ziess, Germany). All types of single chromatid breaks were scored where a clear discontinuity exists. 100 cells were scored per sample. Figure 1 shows a typical metaphase spreads with chromatid aberrations (arrows).

Cell culture for SCE analysis

Blood culture was initiated as described above. 24 hours after culture initiation, 5-bromodeoxy uridine (BrdU, 10 µM, Sigma) was added. Cells were harvested 48 hours after addition of BrdU with the same method described for radiosensitivity study.

Air dried slides were dipped in Hoechst 33258 (100 mg/mL; Sigma) for 10 min in the dark, blotted and placed in a shallow trough containing 2 x SSC (0.3 M NaCl and 0.03 M trisodium citrate) under a UVA source for 4 h. Following exposure, slides were rinsed several times in distilled water and stained in 4% Giemsa. After staining, slides were dipped briefly in distilled water containing a few drops of ammonia to enhance the blue colour of the pale chromatid. Fifty metaphases were scored per sample. A typical photomicrograph of a metaphase showing SCE (arrows) is shown in figure 2.

Statistical analysis

For radiosensitivity study, for each sample the spontaneous yield of chromatid aberrations was scored 100 times and the percentage was calculated by the formula:

\[
\text{Percentage of aberrations} = \frac{\text{Number of aberrated metaphases}}{\text{Total number of metaphases}} \times 100
\]

The results were expressed as the mean ± standard deviation (SD). Statistical analysis was done using analysis of variance (ANOVA) and Bonferroni’s post-hoc tests. The significance level was set at 5% (p < 0.05).

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breaks was subtracted from the yield in irradiated cells to give the induced chromatid break yield. The mean of the aberrations per cell was calculated for each sample. Results were analysed using SPSS (version 17) software (SPSS Inc., Chicago, IL, USA). Paired t-test was used to compare the frequency of chromatid aberrations and SCE within a group and between groups before and after irradiation. The Kolmogorov-Smirnov test showed a normal distribution of data. One way analysis of variance (ANOVA) was used to determine the significant differences between studied groups. P-value of less than 0.05 was considered as significant. Sigma plot 2004 for Windows, version 10.0 was used to draw figures.

RESULTS

The data obtained from normal control and breast cancer patients before and after 1 Gy gamma irradiation is summarized in table 1 and shown in figure 3. Frequency of background and radiation induced chromatid breaks in normal male and female subjects were statistically analyzed and found no significant difference between men and women (p>0.05), therefore data for both sexes were combined in the following statistical analysis. The mean spontaneous yield of chromatid breaks was significantly different between control and cancer patients (p<0.05). However, after gamma irradiation the frequency of chromatid breaks increased dramatically significantly different with unirradiated samples (p<0.01). Also the frequency of breaks in samples from breast cancer patients was significantly higher than normal individuals (p<0.01) (figure 3).

The histograms in figure 4 (A and B) show the distribution of individuals with variable number of radiation induced chromatid breaks. The mean ± 1 SD of the induced frequency of breaks in the control group was used as an arbitrary cut-off point as suggested by Scott et al. (20). As shown in figure 4, 12% of individuals in the control group (6 out of 50) (figure 4A), 47% (28 out of 60) (figure 4B) were regarded as showing elevated radiosensitivity.

Table 2 summarized the results obtained for cells scored for SCE. There was no significant difference between data obtained for men and women in control group (p>0.05), therefore data for both sexes were combined in the following analysis when comparing normal group with breast

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Sex</th>
<th>No. of samples</th>
<th>Mean age (Range)</th>
<th>No. of cells analyzed</th>
<th>Mean background breaks ± SE</th>
<th>Mean gamma rays induced breaks ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Female and male</td>
<td>50</td>
<td>37.4 ± 11 (23-66)</td>
<td>5000</td>
<td>3 ± 0.14</td>
<td>21.1 ± 2.1</td>
</tr>
<tr>
<td>Breast cancer patients</td>
<td>Female</td>
<td>60</td>
<td>46 ± 13.9 (25-76)</td>
<td>6000</td>
<td>± 0.13*</td>
<td>37.7 ± 3.3 **</td>
</tr>
</tbody>
</table>

*Significantly different from control (p<0.05)
** Significantly different from control (p<0.01)
cancer patients. Statistical analysis of the frequency of SCE scored in lymphocytes of breast cancer patients was significantly higher than cells obtained from controls (p<0.05).

**DISCUSSION**

The G₂ assay gives a measure of the frequency of chromatid aberrations in the form of chromatid breaks and gaps present in cells arrested in metaphase following irradiation of peripheral blood lymphocytes or other types of cells in the G₂ phase of the cell cycle. The aberrations resulting from the radiation treatment indicates the radiosensitivity of an individual. The G₂ assay might show association between radiosensitivity and genetic predisposition to cancer indicating individuals with a strong predisposition to cancer show higher chromosomal aberrations hence exhibit higher degree of radiosensitivity (34).

Results presented in table 1 and figure 3 indicate that lymphocytes of breast cancer patients show an elevated frequency of chromatid aberrations compared to lymphocytes obtained from normal controls; hence, more radiosensitivity than control individuals. As seen in figure 4 about 12% of apparently normal individuals (figure 4A) and 47% of breast cancer patients (figure 4B) were radiosensitive. Almost similar results with slight variations were reported previously. Scott et al. reported 9% of normal individuals and 42% of breast cancer patients showed radiosensitivity with the G₂ assay (19, 20). In a study performed in Scotland (UK), chromatid break frequencies were compared for a cohort of previously untreated sporadic breast cancer patients and hospital outpatient controls. In this study 46% of the breast cancer group

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Sex</th>
<th>No. of samples</th>
<th>Mean age ± SD</th>
<th>No. of Cells analyzed</th>
<th>Mean SCE/ cell</th>
<th>Frequency (min-max)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Male</td>
<td>10</td>
<td>38.7 ± 10.37</td>
<td>500</td>
<td>3.54 ± 0.39</td>
<td>2.79 – 3.97</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>20</td>
<td>35.5 ± 8.68</td>
<td>1000</td>
<td>3.35 ± 0.26</td>
<td>2.87 – 3.85</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>30</td>
<td>36.6 ± 9.22</td>
<td>1500</td>
<td>3.41 ± 0.32</td>
<td>2.79 – 3.97</td>
</tr>
<tr>
<td>Breast cancer patients</td>
<td>Female</td>
<td>30</td>
<td>46 ± 11.7</td>
<td>1500</td>
<td>3.67 ± 0.33*</td>
<td>2.88 – 4.17</td>
</tr>
</tbody>
</table>

*Significantly different from control (p<0.05)
showed high radiosensitivity compared to 14% of controls (P< 0.001) (35). Also, with the G2 assay 43% of the breast cancer patients with a known or putative genetic predisposition were found to be radiosensitive (18). Our results are more or less similar to the results reported by Riches et al. (35) both for controls and breast cancer patients.

Although there exists a significant difference between the radiosensitivity of cancer prone patients (individuals with various genetic disorders such as ataxia telangiectasia) and normal control; however, there appears to be a considerable discrepancy in the radiosensitivity of control individuals. Sanford et al. carried G2 assay on two batches of different control groups at different years. They showed a considerable variation between the frequency of chromatid breaks in each study group (34). Since there is a considerable variation between normal control individuals, the relationship between cancer prone individuals and radio-sensitivity might be problematic. Chromatid breaks are direct consequence of double strand breaks in DNA. Therefore defective DNA repair machinery was suggested as a potential susceptibility factor, predisposing women to breast cancer (36). Helzlsouer et al. in a study with G2 assay on several women at high risk of breast cancer and breast cancer patients showed a correlation for suboptimal repair and those individuals at high risk for breast cancer (36). Patel et al. investigated DNA repair proficiency in breast cancer patients by measuring the frequency of chromatid aberrations in terms of disappearance of chromatid breaks over various time intervals in the G2 assay (37). This study in line with previous findings (34, 36) support that DNA repair is defective in individuals predisposed to cancer. To account for high levels of radiosensitivity and cancer predisposition, several experiments were performed indicating that the DNA of cancer prone cells as well as breast cancer patients repair more slowly or produced more breaks than normal individuals (16, 38, 39). These observations support the findings that cells exhibiting enhanced chromatid radiosensitivity are deficient in DNA repair (34, 40, 41). These findings might be indicative that radiosensitivity could be a potential predisposing condition to breast cancer through mutations in low penetrance genes (20, 42) which may be involved in DNA damage repair processing.

Examination of sister chromatid exchanges (SCE) in lymphocytes may be useful for the evaluation of exposure to mutagens/carcinogens. Regarding higher frequency of sister chromatid exchange in lymphocytes of breast cancer patients compared to controls (table 2), there is not a general agreement about a possible association between SCE and cancer. After study of 131 women presented for breast tumor removal, Husum et al. concluded that SCE in lymphocytes is not an indicator of carcinoma of the breast (31). However, women with active breast cancer have shown to have a significantly higher mean SCE frequency than control women (32, 33). Our results also are in line with these reports indicating a higher frequency of SCE per cell in lymphocytes of breast cancer patients compared to normal control. Sister chromatid exchanges reflect an interchange mechanism between sister chromatids of mitotic chromosomes (43). However, background frequency of SCE is influenced by BrdU treatment (44) which is used usually in fluorescence plus Giemsa method for SCE detection (45), lymphocyte counts (46) and possibly various environmental factors such as smoking. Therefore SCE might not be a good predictive marker for screening cancer prone individuals including those at risk of breast cancer.

Both high frequency of radiation induced chromatid breaks and background frequency of SCE in lymphocytes of breast cancer patients might be due to the genome instability of cells. Genome instability has also been described for various hereditary
cancers including breast cancer (17, 18). Therefore genome instability might be the underlying mechanism of various cellular responses leading to neoplastic development.

In conclusion it seems that G2 assay for assessment of in vitro radiosensitivity of peripheral blood lymphocytes has potential for screening individuals at risk of breast cancer.

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