Risk assessment in first degree female relatives of breast cancer patients using the alkaline Comet assay

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First degree female relatives (FDFRs) of breast cancer patients have been reported to have a 2- to 3-fold increase in breast cancer risk as compared with the general population. Assessment of genetic instability (DNA damage and repair efficiency) is an important parameter concerning mutagenesis and carcinogenesis. In an attempt to identify individuals at high risk of breast cancer in the FDFRs of breast cancer patients, two tests were used: the alkaline Comet assay on leucocytes and the micronucleus test (MNT) on buccal epithelial cells. In addition to FDFRs, two other categories of subjects were included: breast cancer patients and controls. The Comet assay was used to study basal DNA damage, DNA susceptibility to a mutagen (N-methyl N-nitro N-nitrosoguanidine) and DNA repair efficiency. In addition, the MNT served as an indicator of chromosome breakage/aneuploidy. A significant increase in DNA damage (basal and after treatment with a mutagen, as well as after allowing repair to take place) and micronucleus frequency was observed from controls to FDFRs and from FDFRs to breast cancer patients. There was considerable variability in the subjects with respect to both of these parameters. Outliers identified among the FDFRs based on 3 SD limits of DNA damage and micronucleus frequency were considered as high risk individuals.

Introduction

Breast cancer is the second most frequent site of cancer in India. Its incidence in India is estimated to be 24 per 100 000 (1). Certain women appear to be at an increased risk (2). As compared with the general population, the first degree female relatives (FDFRs) of breast cancer patients have been reported to have a 2- to 3-fold increase in breast cancer risk, which could be due to shared genetic and/or environmental components (3). Consequently, it would be expected that considerable heterogeneity exists for breast cancer among FDFRs. To date, no biomarker has been available which could be used to identify individuals at high risk in FDFRs. This study was undertaken to look for such a biomarker(s).

The carcinogenic process is generally associated with genetic instability, and to evaluate genetic instability, the use of sensitive detection techniques are required. The information thus obtained will be helpful in identifying people who are at higher risk (4).

A number of biomarkers have been used to analyse genetic instability. The most extensively used biomarkers involve the cytogenetic evaluation of chromosome aberrations and/or sister chromatid exchanges in mitogen-stimulated cells. Although cytogenetic methods provide information concerning DNA damage at the single cell level, they are usually limited to the proliferating cell population, require moderate volumes of blood, involve extended processing times and are relatively labor intensive. Some biochemical and immunological techniques, like the alkaline or neutral gel elution methods (5–7), have also been used. However, these techniques use pooled cell populations and, thus, are unable to provide critical information about intercellular differences in DNA damage and repair.

The alkaline single cell gel electrophoresis or alkaline Comet assay is one such sensitive technique that allows the detection of DNA damage and repair in G0 cells, at the single cell level and in virtually any eukaryotic system that can be dispersed as a single cell suspension (8). Moreover, this test is less expensive and results can be obtained within a relatively short time after sampling (i.e. within a few hours). The alkaline Comet assay has been used for the first time, in the present study, in risk assessment of FDFRs. To substantiate our results and to provide a cytogenetic parameter, the micronucleus test (MNT), was also carried out.

Materials and methods

Subjects

Three hundred and ninety-seven female subjects comprising 188 FDFRs of breast cancer patients (age range 18–70 years), 88 newly diagnosed untreated breast cancer patients (age range 18–70 years) and 121 healthy controls (age range 18–70 years) with no family history of any type of cancer were included in the study. All the subjects were non-smokers and non-alcoholics. Personal data on each subject were collected in terms of age, laterality of the affected breast, family history, menstrual history, menopause status, parity, age at first pregnancy and diet (frequency and quantity of vegetarian and non-vegetarian food and tea, coffee and alcohol intake).

Alkaline Comet assay

About 150 µl of peripheral blood was collected from each subject into a heparinized microtube. After testing for leucocyte viability using the trypan blue exclusion test each blood sample was divided into three aliquots. The mean percentage viability for each group of subjects was found to be 91.7% (FDFRs and controls 95%; breast cancer patients 85%). Each aliquot of peripheral blood was suspended in 350 µl of RPMI 1640 medium with 20% fetal calf serum. The first aliquot was used to study the basal DNA damage (BDD). The second and third aliquots were incubated with 0.025 µM final concentration of N-methyl N-nitro N-nitrosoguanidine (MNNG) in the dark at 37°C for 30 min. The second aliquot was used to study an individual’s susceptibility to a mutagen treatment. The third aliquot was further incubated at 37°C in a fresh mixture of 350 µl RPMI 1640 medium with 20% fetal calf serum for 2 h to study repair efficiency (for details of MNNG treatment and post-treatment repair see ref. 9). All three aliquots were processed for the Comet assay.

The Comet assay was carried out as described by Singh et al. (10) with slight modifications (9). In brief, the washed cells were suspended in 110 µl

Abbreviations: BDD, basal DNA damage; FDFRs, first degree female relatives; MNNG, N-methyl N-nitro N-nitrosoguanidine; MNT, micronucleus test.
of low melting point agarose (0.5% in phosphate-buffered saline) and layered on a fully frosted slide, precoated with 140 µl of normal melting point agarose (0.75% in phosphate-buffered saline). Finally, 110 µl of low melting point agarose was layered on top. The slides thus prepared in duplicate were left overnight at 4°C in freshly prepared lysing solution (2.5 M NaCl, 100 mM Na2 EDTA, 10 mM Tris, 1% Triton X-100, 10% dimethyl sulphoxide added fresh). The cells were treated with alkaline buffer (1 mM Na2 EDTA and 300 mM NaOH, pH 13) for 20 min to allow denaturing and unwinding of the DNA as well as expression of alkali-labile sites. This was followed by electrophoresis in the same buffer at 20 V (0.8 V/cm) and 300 mA for 25 min to allow damaged DNA/fragments to migrate towards the anode.

The cells were neutralized with 0.4 M Tris buffer (pH 7.5) stained with 60 µl of aqueous ethidium bromide (20 µg/ml) and examined under a fluorescence microscope. An undamaged cell appeared as a nucleoid and a cell with damaged DNA appeared as a comet.

The length and the maximum diameter of the comet were measured using an ocular micrometer affixed in the eyepiece. The comet tail length, which is an estimate of DNA damage for each cell, was calculated as the difference between the length and maximum diameter of the comet. Fifty cells per treatment were scored at random for each individual.

**Microscope test (MNT)**

In addition to the alkaline Comet assay, the MNT was also carried out on the buccal epithelial cells of 70 FDFRs (age range 18–70 years), 30 breast cancer patients (age range 18–70 years) and 46 controls (age range 18–70 years). All subjects were selected at random. Buccal epithelial cells were collected by gently scraping the buccal mucosa with a moistened tongue depressor. The cells were smeared on a precleaned slide, fixed in methanol and stained with 2% giemsa. One thousand cells were scored for each subject and the percentage of cells with micronuclei was calculated.

**Statistical analysis**

The slides were coded at the time of preparation and scoring. They were decoded before statistical analysis for comparison. ANOVA and Student’s t-test were carried out to compare the mean comet tail length values between and within the groups. Inter-individual variability was also studied by frequency distribution based on the percentage of individuals in each group against mean comet tail length. Multiple linear regression analysis was carried out to determine the effect of various confounding factors. The frequencies, outliers were identified based on 3 SD limits or values that appear outside the normal distribution curve (11). Samples were coded at the time of collection and decoded after statistical analysis to perform a blind study.

**Results**

A total of 19 850 cells were analysed for DNA damage using the alkaline Comet assay in FDFRs, breast cancer patients and controls. The BDD level, that after post-exposure repair significantly increased from controls to FDFRs and from FDFRs to breast cancer patients (Table I).

A total of 146 000 buccal epithelial cells were analysed for micronuclei in FDFRs, breast cancer patients and controls. There was a significant increase in the frequency of micronuclei from controls to FDFRs and from FDFRs to breast cancer patients (Table II).

The frequency distribution showed that the range of inter-individual variability increased from controls to FDFRs and from FDFRs to breast cancer patients. The percentage of individuals with highly damaged cells again increased from controls to FDFRs and from FDFRs to breast cancer patients (Figures 1–3).

From the present study, out of 188 FDFRs studied four individuals (2.13%) were identified as high risk individuals based on 3 SD limits.

Analysis of the possible confounding factors influencing DNA damage in FDFRs has shown that the identified high risk individuals were all pre-menopausal individuals. All of them had a pre-menopausal affected relative (sister and/or daughter) with right side affected breast cancer. Moreover, two of them also had a family history of breast cancer with an affected sister and/or daughter. Other confounding factors such as age of the individual, parity, menstrual history, age at first pregnancy and diet did not influence the DNA damage.

### Table I. Inter-group comparison of mean DNA damage (mean comet tail length in µm)

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of cases</th>
<th>DNA damage (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>BDD Mean ± SD</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Range</td>
</tr>
<tr>
<td>(A) Breast cancer patients</td>
<td>88</td>
<td>16.38 ± 6.54</td>
</tr>
<tr>
<td>(B) FDFRs</td>
<td>188</td>
<td>6.09 ± 4.70b,c</td>
</tr>
<tr>
<td>(C) Controls</td>
<td>121</td>
<td>2.38 ± 1.81b,c</td>
</tr>
</tbody>
</table>

*a* A versus B, *P* < 0.001.

*b* A versus C, *P* < 0.001.

*C* B versus C, *P* < 0.001.

### Table II. Inter-group comparison of micronucleus frequency

<table>
<thead>
<tr>
<th>Group</th>
<th>Micronucleus frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of cases</td>
</tr>
<tr>
<td>(A) Breast cancer patients</td>
<td>30</td>
</tr>
<tr>
<td>(B) FDFRs</td>
<td>60</td>
</tr>
<tr>
<td>(C) Controls</td>
<td>46</td>
</tr>
</tbody>
</table>

*a* A versus B, *P* < 0.001.

*b* A versus C, *P* < 0.001.

*c* B versus C, *P* < 0.001.

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**Fig. 1.** Frequency distribution of individuals against mean comet tail length (µm) at basal level in peripheral blood leucocytes.
Discussion

Alkaline Comet assay

Increased genomic instability, either inherent or induced by external agents (a mutagen or a carcinogen), has been considered as a primary event leading to neoplastic transformation (12,13). Hence, in this study, the genomic instability was studied at basal level, after in vitro MNNG treatment and after 2 h incubation for repair.

Basal DNA damage (BDD)

The level of DNA instability of an individual at baseline level is likely to play a critical role in cancer predisposition and progression. In the present study, the DNA damage significantly increased from controls to FDFRs and from FDFRs to breast cancer patients (Table I and Figure 1). This increase may be due to progressive genetic instability. We observed that the FDFRs showed ~2.5 times higher DNA damage as compared with the controls. From our study it is evident that the FDFRs in the breast cancer families were at an increased risk of breast cancer as compared with control families. Schwartz et al. (14) also reported a relative breast cancer risk of 1.8–4.2 in breast cancer families as compared with control families.

In this study, maximum DNA damage was observed in the breast cancer patients. In support of our observations, as compared with the controls a significant increase in DNA damage was observed in the bladder washings of bladder cancer patients (15) as well as in the leucocytes and cervical epithelial cells of cervical cancer patients (9), using the Comet assay. Similarly, an increase in chromosomal abnormalities, like deletions, breaks and gaps, has been reported in the lymphocytes of breast cancer patients as compared with controls (16,17). In contrast, Kovacs and Almendral (18) and Jyothish et al. (19) observed no difference in the level of spontaneous DNA synthesis and chromosome aberrations between FDFRs and cancer patients. However, in our study we observed a significant difference in basal DNA damage between basal DNA damage between breast cancer patients and FDFRs and between FDFRs and controls.

Susceptibility to mutagen treatment

MNNG (an alkylating mutagen) is known to induce single-strand DNA breaks as detected by the Comet assay (20,21). In the present study also, DNA damage was significantly enhanced after in vitro treatment of leucocytes with MNNG in the breast cancer patients, FDFRs and controls (Table I). Assuming the basal DNA damage in the controls as spontaneous damage, FDFRs and breast cancer patients showed higher susceptibilities to mutagen treatment than the controls (Table I and Figure 2). Similarly, Jyothish et al. (19) and Jaloszynski et al. (22) reported that the lymphocytes of breast cancer patients are hypersensitive to bleomycin (a radiomimetic agent)-induced chromosome damage.

Repair efficiency

The susceptibility of a person to different carcinogens is the net result of that person’s defences against agents that can damage DNA and the ability of his/her cells to accurately repair such damage (23). Chromosome instability syndromes like ataxia telangiectasia, xeroderma pigmentosum, Bloom syndrome and Fanconi anaemia are characterized by defective DNA repair and a high incidence of cancer (24), suggesting that DNA repair deficiency is a prerequisite for carcinogenesis (25,26). In the present study, when DNA repair was monitored the damage regressed in all three groups (Table I and Figure 3). The persisting DNA damage in the FDFRs and breast cancer patients as compared with the controls indicated reduced DNA repair synthesis in these groups. Decreased DNA repair synthesis in leucocytes has also been reported in breast cancer patients (22,27,28) and healthy FDFRs of breast cancer patients (18,29).

Micronucleus frequency

Rosin and German (30) reported an increased frequency of micronuclei in Bloom syndrome homozygotes as compared to Bloom syndrome heterozygotes and controls in buccal mucosal and urinary tract mucosal cells (two widely separated epithelia which are not target tissues for the occurrence of cancer in Bloom syndrome). Homozygotes are susceptible to cancers of the skin, breast and lung and also leukaemia (31). In this study also, when the MNT was carried out on buccal epithelial cells (a non-target tissue), a significant increase in micronucleus frequency was seen from controls to FDFRs and from FDFRs to breast cancer patients (Table II).

Inter-individual variation

Inter-individual variation in terms of chromosomal aberrations has been reported in normal subjects and cancer patients, when their lymphocytes were cultured under normal circumstances or under environmental stress (32–34). Inter-individual differences in mutagen sensitivity (35,36) and DNA repair activity (37–39) have been reported, with some individuals exhibiting increased sensitivity and decreased ability to repair the DNA damage induced by genotoxicants (40–42). In the present study also, inter-individual variability in terms of DNA damage was observed at the basal level and after mutagen treatment, as well as after allowing for repair to take place in the leucocytes of breast cancer patients, FDFRs and controls. Moreover, we noted that the range of inter-individual variability and the percentage of individuals with highly damaged cells increased from controls to FDFRs and from FDFRs to breast cancer.

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patients (Figures 1–3). The reason for this variation is multifactorial and it results from the interplay of both intrinsic and extrinsic factors like differential cellular responses to mutagen action, DNA repair and replication (43–45).

**High risk individuals**

The best long-term prospects for a reduction in the number of deaths due to cancer lie with screening for high risk individuals and prevention rather than with therapy of the advanced disease. Screening efficiency could be improved by employing techniques with high sensitivity.

Susceptibility to cancer, in general, is characterized by high DNA damage, which is the result of low repair capacity. To identify high risk individuals, in the present study four parameters were considered: (a) basal DNA damage; (b) susceptibility to mutagen; (c) repair efficiency; (d) micronucleus frequency. A score of 1 was given to the outliers crossing 3 SD limits for any of these parameters. From this preliminary screening, out of the total of 188 FDFRs screened, 23 were identified with a minimum score of 1. Twelve of them were outliers for only one parameter, seven of them for a score of 2, four of them for a score of 3 and none with a score of 4. Since, it would be appropriate to consider more than two parameters in risk assessment studies (9), in this study the FDFRs with a minimum score of 3 (i.e. outliers for three parameters) were considered as high risk individuals. From this study four (2.13%) such individuals were spotted. Comparable with our results, Parkin et al. (1) have also reported that the lifetime cumulative breast cancer incidence in India is 2.11% (averaged over the lifetime cumulative incidences of Banglaore, Barshi, Bombay, Karungapally, Madras and Trivandrum).

According to our working hypothesis, these individuals were considered to be at a higher risk with an increased predisposition for breast cancer in the long term. Moreover, all the identified high risk FDFRs in our study showed increased susceptibility to mutagens and reduced repair efficiency. In support, Jalszynski et al. (22) also reported that increased long-term overall sensitivity may be important factors for breast cancer development.

Since the latent period for cancer may be long, it is difficult to see the outcome in such studies. However, wherever possible, efforts are being made to follow up the high risk individuals. For example, in a landmark study, the Nordic study group has verified the association of the magnitude of chromosome damage with cancer risk (46).

**Confounding factors**

In a breast cancer family, the relative risk for breast cancer is dependent on various factors. Several epidemiological studies have brought out the association of family history, young age at diagnosis (<45 years), a pre-menopausally affected relative (mother and/or sister) and laterality (right side affected breast) with breast cancer risk (47,48).

Analysis of the possible confounding factors in the present study showed that DNA damage and micronucleus frequency were influenced by the age of the subject, her menopausal status, age of the affected relative and the affected relative’s menopausal status. When possible confounding factors were analysed in the high risk FDFRs, all four individuals identified as at high risk were pre-menopausal (<50 years), having a pre-menopausal relative with right side affected breast; two of them had a positive family history of breast cancer (more than one relative affected) with an affected sister and/or an affected daughter.

**Conclusions**

In the present study, an effort has been made to biomonitor high risk FDFRs based on DNA damage and repair using the alkaline Comet assay and MNT. Although, the MNT and alkaline Comet assay represent different end-points, the results obtained from both of them were very similar. From the present study it is evident that the FDFRs are at an increased risk for breast cancer as compared with the control population. However, the effects of critical inherited alterations leading to cancer are latent for an extended period (49). Hence, long-term, large-scale follow-up studies in the high risk FDFRs would be required to confirm the conclusions of this study. In the meantime, such follow-up studies using animal models are underway in our laboratory. Data presented here suggest that the alkaline Comet assay and MNT can determine important biomarkers in population monitoring to identify individuals at high risk due to their simplicity and sensitivity.

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**References**


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