Induced Micronucleus Frequencies in Peripheral Lymphocytes as a Screening Test for Carriers of a BRCA1 Mutation in Breast Cancer Families

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ABSTRACT

Enhanced sensitivity to the chromosome-damaging effects of ionizing radiation is a feature of many cancer-predisposing conditions. It has been suggested that women with breast cancer are deficient in the repair of radiation-induced DNA damage. We have now investigated whether mutagen sensitivity is related to mutations in the breast cancer gene BRCA1. We studied the induction and repair of DNA damage in lymphocytes of women from familial breast cancer and breast and ovarian cancer. The mutagens used were gamma-irradiation and hydrogen peroxide and the DNA effects were determined with the micronucleus test and the comet assay. Women with a BRCA1 mutation (n = 12) and relatives without the familial mutation (n = 10) were compared to controls (i.e., healthy women without family history of breast or ovarian cancer; n = 17). Our results indicate a close relationship between the presence of a BRCA1 mutation and sensitivity for the induction of micronuclei. Compared to a concurrent control, 10 of 11 women with a BRCA1 mutation showed elevated radiation sensitivity. Of the 10 related women without the familial mutation, only 2 had clearly enhanced micronucleus frequencies. In addition to the sensitivity toward gamma-irradiation, hypersensitivity toward hydrogen peroxide was also observed, indicating that the mutagen sensitivity is not solely due to a defect in the repair of DNA double strand breaks. In contrast to the results with the micronucleus assay, we found no significant difference between women with and without a BRCA1 mutation with respect to the induction and repair of DNA damage in the comet assay. This finding suggests a normal rate of damage removal and points to a disturbed fidelity of DNA repair as a direct or indirect consequence of a BRCA1 mutation. Our results support the usefulness of induced micronucleus frequencies as a biomarker for cancer predisposition and suggest its application as a screening test for carriers of a BRCA1 mutation in breast cancer families.

INTRODUCTION

The identification of breast and/or ovarian cancer susceptibility genes known as BRCA1 and BRCA2 allows genetic testing in high-risk families despite various unresolved problems related to the reliability, sensitivity, and predictive value of testing, as well as the safety and efficacy of monitoring procedures and preventive strategies (1). One critical aspect is the enormous number of mutations reported thus far in breast cancer genes.3 This high number of mutations means that full sequencing of the genes is the only reliable way to screen them (2). However, such sequencing is time-consuming, difficult, and costly. Furthermore, not every mutation associated with breast and/or ovarian cancer can be detected, because the change lies outside the protein-coding regions of the genes or is due to a large genomic rearrangement. Comparison of linkage with mutation data for the families in the International Breast Cancer Consortium suggests that up to 30% of mutations escape detection (3). On the other hand, the interpretation of a positive sequencing test result also has limitations. Because this test assays for DNA sequence variation, one cannot always be certain that a particular variant is significant in terms of risk. Besides, one-third of all breast cancers that seem to run in families do not show linkage to either BRCA1 or BRCA2, indicating the involvement of one or more further breast cancer gene(s) in familial breast cancer that are yet to be discovered (3, 4). For these reasons, additional tests indicating predisposition for cancer are desirable. It has been suggested that women with breast cancer are deficient in the repair of radiation-induced DNA damage (5), and it has more recently been shown that radiation-induced MN4 induction in lymphocytes identifies a high frequency of radiosensitive cases among breast cancer patients (6). In the latter study, 31% (12 of 39) of breast cancer patients had elevated radiation sensitivity, compared with 5% (2 of 42) of healthy controls. However, the study could not show whether the observed radiation sensitivity is a heritable trait and associated with the known BRCA mutations. We therefore evaluated the utility of the MNT with peripheral lymphocytes as a screening test for BRCA mutations in breast cancer families.

MATERIALS AND METHODS

Blood Samples. Heparinized blood samples were obtained by venepuncture from 22 members of 13 families with a familial BRCA1 mutation, ages 23–58 years (mean, 42 ± 10). Our study was part of a multicenter study on familial breast cancer, and the criteria for the participation in the study were as follows: (a) families with two or more women with breast and/or ovarian cancer (and at least one woman less than 50 years of age at the time of diagnosis in families with only two affected women); (b) families with at least one woman with bilateral breast cancer or breast and ovarian cancer; and (c) families with at least one woman with breast cancer diagnosed before the age of 30 years or with ovarian cancer diagnosed before the age of 40 years.

For controls, we selected 17 age-matched women (25–53 years; mean, 36 ± 9) without any family history of cancer. All blood donors gave informed consent to participate in this study. Freshly collected blood was used directly (for the comet assay) or diluted in chromosome medium (for the MNT). Blood samples were exposed to cobalt-60 gamma rays at 4 Gy/min. After irradiation, blood was brought to the laboratory, where the tests were started within 5 min. During this time period, blood samples for the comet assay were kept on ice to inhibit repair of induced DNA damage. Hydrogen peroxide (Sigma Chemical Co., Munich, Germany) was diluted in distilled water. It was added to the blood cultures for the induction of MN at the start of the cultures and maintained until cell harvest.

MNT. The MNT was performed as described earlier (7). Blood (0.3 ml) was added to 3 ml of chromosome medium A (Life Technologies, Inc.), supplemented with 2% phytohemagglutinin-L (Life Technologies, Inc.) and incubated at 37°C. Cytochalasin B (Sigma) was added to the cultures at a final concentration of 6 µg/ml, 44 h after phytohemagglutinin stimulation. Cultures were harvested 24 h later, giving a total culture time of 68 h. Cells were harvested by centrifugation, treated with a hypotonic solution (0.56% KCl), fixed once with methanol/glacial acetic acid (3:1) mixed with an equal amount of 0.9% NaCl, and then fixed twice with methanol/glacial acetic acid. Air-dried slides were stained with acridine orange (125 µg/ml in phosphate buffer). The

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4 The abbreviations used are: MN, micronucleus (micronuclei); MNT, micronucleus test; dsh, double strand break; sbs, single strand break; A-T, ataxia telangiectasia.

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frequency of MN was determined by analyzing 1000 binucleated cells from coded slides.

Comet Assay. Aliquots of 5 µl of blood were mixed with 120 µl of low-melting-temperature agarose (0.5% in PBS) and added to microscope slides (with frosted ends) that had been covered with a bottom layer of 1.5% agarose. Slides were processed as described earlier (8) using a time of alkali denaturation and electrophoresis (0.86 V/cm) of 25 min each. Images of 50 randomly selected cells stained with ethidium bromide were analyzed from each coded slide. Measurements were made by image analysis (Perceptive Instruments), determining the median tail moment of the 50 cells.

Statistical Analysis. Differences between mean values were tested for significance (P < 0.01) using Student’s t test.

Direct Sequencing of BRCA1. Genomic DNA was isolated from blood samples (EDTA) using the Blood & Cell Culture DNA kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The 22 coding exons of the BRCA1 gene were analyzed in 36 fragments with M13-tailed primers. Exon 11 was split into 14 overlapping fragments, using published primer sequences (9, 10), and primers for the exons 7, 9 and 16 were newly designed (Lasergene software; DNASTAR, Madison, WI). Amplifications were carried out using a GeneAmp PCR system 9600 (Perkin-Elmer, Foster City, CA). Cycle-sequencing was performed in sense and antisense direction by the ABI 877 Integrated Thermal Cycler (Perkin-Elmer) using the Ready Reaction Big Dye primer cycle sequencing kits with AmpliTaq FS (Perkin-Elmer) according to the manufacturer’s instructions. One µl of the samples was loaded onto a 4.5% polyacrylamide gel (29:1) containing 6 M urea on an ABI 377A DNA sequencer (Perkin-Elmer). Sequence data files were analyzed using Factura software (Perkin-Elmer). After processing, the forward and reverse sequence files were imported in Sequence Navigator software (Perkin-Elmer) and aligned to the wild-type reference files.

RESULTS AND DISCUSSION

Our study was performed in the course of a multicenter breast cancer study in Germany that was set up to evaluate diagnostic and therapeutic standards for familial breast cancer. In this study, genetic testing for BRCA1 and BRCA2 mutations was offered to women with defined family histories of cancer. We had access to a limited number of subjects from 13 families with familial breast cancer or breast and ovarian cancer with a BRCA1 mutation. We performed the MNT with and without gamma-irradiation of the blood samples and set up parallel cultures from healthy controls. Fig. 1 shows examples of four families carrying four different BRCA1 mutations (two frame-shift mutations, one nonsense mutation, and one missense mutation). From these four families, we tested more than one subject. The induced MN frequencies (i.e., MN frequency after irradiation with 2 Gy minus MN frequency of the unirradiated culture of the same subject) are given for the subjects from the breast cancer families and for concurrent controls. It is obvious that the carriers of a familial BRCA1 mutation exhibit strongly increased MN frequencies, whereas first-degree relatives without the mutation have induced MN frequencies in the range of the controls. It can also be seen (Fig. 1D) that a young woman without a diagnosed cancer but carrying the familial BRCA1 mutation clearly shows radiation sensitivity. These findings indicate a correlation between BRCA1 mutations and radiation sensitivity in the MNT. There was more variation among the spontaneous MN frequencies of test persons (between 7 and 37 MN/1000 BNC, with a mean of 17.8 and one outlier with 161 MN/1000 BNC) than there was among controls (between 6 and 17 MN/1000 BNC, with a mean of 8.5). Higher spontaneous frequencies could be explained in some cases by a previous radiation therapy and are in accordance with earlier findings in breast cancer families (6). However, elevated radiation sensitivity was not a consequence of therapy but was also observed in cancer patients without radiation therapy and normal spontaneous MN frequencies. Fig. 2 summarizes all data obtained thus far from breast cancer families and controls. Subjects were ordered according to their induced MN frequencies in three groups: controls (Fig. 2A), women with BRCA1 mutations from families with breast cancer or breast and ovarian cancer (Fig. 2B), and women without BRCA1 mutation from families with familial breast cancer or breast and ovarian cancer (Fig. 2C). We determined the mean of the MN frequencies in controls; indicated on the figure is the +2 SD level of the control group, which was used as an arbitrary cutoff for sensitive subjects by Scott et al. (6). Using this cutoff, two women without the familial BRCA1 mutation revealed clearly increased MN frequencies (Fig. 2B). These women were from two different families and both of them suffered from breast cancer. One possible explanation is that they carried undetected cancer-related mutations. The comparison of the control group (Fig. 2A) with the group of women carrying a BRCA1 mutation (Fig. 2C) indicates a clearly increased
mean MN-frequency in the latter. Using the +2 SD cutoff, 5 of 12 subjects with *BRCA1* mutations did not fall into the group of sensitive subjects. However, the difference between the induced MN frequencies of mutation carriers and the concurrent control (Fig. 3) revealed that 10 of 11 women had clearly higher MN frequencies than the control and would have been classified as sensitive in our test protocol. One woman with a *BRCA1* mutation could not be evaluated in this way because the parallel control could not be analyzed due to a technical problem. However, this woman had an induced MN frequency of 373, which we would consider as being high and indicating sensitivity under our test conditions. Furthermore, her sister carried the same mutation and also showed radiation sensitivity. Until now, we did not have the chance to clarify this case in a repeated test. Fig. 3 also indicates that our group of radiation sensitive women includes seven different *BRCA1* mutations in various regions of the gene. Only one woman with a *BRCA1* mutation (Fig. 3, column 6) had no elevated induced MN frequency in comparison to the control. One possible explanation for a normal MN frequency despite a *BRCA1* mutation might be that the mutation does not affect the function of the *BRCA* gene product. However, in our case, the woman carried a mutation (C4302T) that was also found in another family, where there was an association with high MN frequencies (Fig. 3, column 7).

Furthermore, this mutation is a known nonsense mutation, which should lead to a misfunctional protein. We received a second blood sample from this particular patient, but also, the second independent test gave no indication for radiation sensitivity. Larger studies are necessary to show whether the choice of the mean + 2 SD of controls is reasonable for the identification of radiation-sensitive subjects. In our hands, a parallel control enabled a better discrimination. This might at least partly be explained by a relative high variation between independent tests. One possible reason for this variation might be the kind of gamma radiation. The high dose-rate exposure, as used in our tests, required a short irradiation time that might be subject to minor but possibly relevant variation. The use of an optimized radiation source (i.e., with a lower dose rate) might avoid this problem. Another reason for variation might be the quality of the cell preparation. We observed that slides with clear separation of cells and optimal differential fluorescent staining tend toward higher MN counts. We believe that even in highly experienced laboratories, some degree of methodological variation is unavoidable. However, the direct comparison to a concurrent control reveals a difference between about 100 and 200 MN per 1000 cells in 10 of 11 cases (Fig. 3). Such a difference could be taken as an alert and could, for example, justify further testing for mutations.

We also found increased radiosensitivity in two women with *BRCA2* mutations from two families with familial breast cancer (data not shown), indicating that the radiation sensitivity may result from mutations in various genes related to heritable cancer predisposition. Earlier studies already demonstrated increased radiosensitivity in the MNT of lymphocytes from individuals with A-T and A-T heterozygotes (11).

Taken together, our findings suggest that the determination of the radiation-induced MN frequency is a simple, rapid, inexpensive, and sensitive test for the identification of carriers of a *BRCA* mutation in families with familial breast and breast or ovarian cancer. In contrast to sequencing, which only assays for DNA sequence variation, the MNT determines a cellular phenotype and thus represents an indicator of a biological effect. Therefore, the MNT might be useful as a prescreening test for familial *BRCA* mutations, especially in cases in which sequencing is not considered due to the fact that the *a priori* risk is low or the relatives with cancer already died. Possibly, the MNT might also help to decide whether a new unclassified *BRCA* variant is biologically significant because it leads to the cellular phenotype of radiosensitivity. However, the relationship between radiosensitivity and cancer risk remains to be established in future investigations.

In an attempt to elucidate the biological basis of the radiation sensitivity, we comparatively tested radiation sensitivity and sensitivity toward hydrogen peroxide in the MNT, and we compared the radiation sensitivity in the MNT with effects on induction and repair of DNA damage in the comet assay. A role of *BRCA1* in DNA repair is suggested by various findings including a specific interaction with Rad51 protein and p53 pathways (for a review, see Ref. 12). However, not much is known about the genetic consequences of a *BRCA1* mutation on the cellular level. We had the chance to perform comparative tests with blood from four women with breast cancer and a familial *BRCA1* mutation, using treatment with gamma irradiation (Fig. 4A) or H₂O₂ (Fig. 4B), respectively. It can be seen that the mutagen sensitivity is not limited to gamma irradiation but also found after H₂O₂ treatment. Whereas treatment with H₂O₂ (2 × 10⁻³ M) has only a marginal effect on the frequencies of MN in normal controls, the same treatment leads to a clear induction of MN in subjects with a *BRCA1* mutation. Because, in contrast to ionizing radiation, H₂O₂ does not induce DNA dsbs in abundance (13), it can be concluded that the mutagen sensitivity of the cells with a *BRCA1* mutation is not
limited to DNA dsbs. It has recently been shown that mouse embryonic stem cells deficient in \( \text{BRCA1} \) are hypersensitive to ionizing radiation and \( \text{H}_2\text{O}_2 \) (14). However, this sensitivity, which has been explained by a defect in the ability to carry out transcription-coupled repair of oxidative DNA damage, was only found in cells with two inactivated \( \text{BRCA1} \) alleles. Our results with \( \text{H}_2\text{O}_2 \) support the idea that \( \text{BRCA1} \) is involved in the repair of oxidative DNA damage, but in contrast to murine cells, human lymphocytes apparently already express the hypersensitivity in the heterozygous state. To further test whether the induction and/or repair of DNA ssbs, a major oxidative DNA lesion, is impaired in cells with a \( \text{BRCA1} \) mutation, we performed comparative tests with the comet assay. The comet assay (single cell gel electrophoresis) is a sensitive test for measuring the induction and repair of DNA damage. In its alkaline version, it is especially sensitive for the detection of DNA ssbs and other oxidative DNA damage (8). Our comparative test with cells from four patients with \( \text{BRCA1} \) mutations and controls did not indicate a significant difference in the induction and repair of gamma radiation-induced DNA damage in the comet assay. We found considerable interindividual variation among patients and controls. Both groups showed a similar induction of DNA effects (tail moment) immediately after irradiation and a similar decrease (about 50–60%) as an indicator of DNA repair 1 h after irradiation. Each of the same patients clearly exhibited sensitivity to irradiation in the MNT (Fig. 5A). These results indicate that the comet assay is not suited to detect individual mutagen sensitivity associated with a \( \text{BRCA1} \) mutation. Although it cannot be excluded that a difference can be detected on the group level (15), the comet assay is obviously less suited than the MNT for screening for familial \( \text{BRCA} \) mutations. Our comet assay results resemble those obtained with cells from patients with A-T and A-T-like Chinese hamster cells (16, 17) and suggest that a \( \text{BRCA1} \) mutation might not mainly affect the induction of damage or the speed of its removal but might reduce the fidelity of repair. This idea is in accordance with the elevated MN frequencies, which can be explained as a result of incorrect DNA repair.

In summary, our study suggests that \( \text{BRCA} \) mutations lead to mutagen sensitivity of lymphocytes and that the MNT is a valid test for the detection of this sensitivity and a good predictor for \( \text{BRCA} \).
mutations in subjects from families with familial breast and/or ovarian cancer. More extended studies will have to show whether the MNT can be used as a screening test for BRCA mutations in women with family history of breast cancer. Its simplicity, rapidity and sensitivity could make the MNT a valuable tool for the evaluation of heritable cancer predisposition in addition to sequencing and other methods of mutation detection with their known problems and limitations. Our results also indicate that the elevated MN frequencies are not solely due to DNA-dsb but also result from DNA ssbs and/or other oxidative DNA damage. It is suggested that the elevated MN frequencies are a consequence of faulty DNA repair.

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