Denaturing Gradient Gel Electrophoresis-based Analysis of Loss of Heterozygosity Distinguishes Nonobvious, Deleterious BRCA1 Variants from Nonpathogenic Polymorphisms, Miguel de la Hoya, Eduardo Díaz-Rubio, and Trinidad Caldes

BRCA1 testing and genetic counseling services are offered to families with histories of breast and/or ovarian cancer. The available screening methods to detect germ-line BRCA1 mutations are expensive and time consuming because the gene is large, prevalent BRCA1 mutations are not found (except in ethnic communities), and mutations are scattered throughout the coding sequence.

Gene screening often detects a BRCA1 variant that does not imply a frameshift or a splicing alteration but represents a missense mutation not previously reported or registered in the Breast Cancer Informative Core Database. To address the question of whether these mutations represent new cancer-predisposing mutations or rare polymorphisms, one must consider characteristics such as absence of the variant in a control group of sufficient size, cosegregation with cancer in some families, and occurrence in a highly conserved protein sequence or in a putative functional domain. These considerations imply the study of large-pedigree families, which very often are not available and the conclusions of which are not always compatible with genetic counseling practice. As an indication of these limitations, only BRCA1 missense mutations that abolish the BRCA1 C-terminal transcriptional activity in a transfection assay or disturb the RING-finger domain structure have been defined as cancer-predisposing mutations. The lack of complete understanding of BRCA1 makes it difficult to design a reliable BRCA1 functional test similar to those for other tumor suppressor genes such as p53 (14).

BRCA1 is a classical tumor suppressor gene that follows Knudson’s two-hits hypothesis. BRCA1 somatic mutations have not been detected in sporadic breast cancer and are very uncommon in sporadic ovarian cancer. These findings indicate that selective retention of one BRCA1 allele in a breast or ovarian tumor is a good predictor of the cancer-predisposing role of this allele.

Very often, index cases from families under study in genetic counseling services are women already diagnosed as having breast or ovarian cancer, and fresh or paraffin-embedded tissue samples from their tumors are readily available. In these cases, a loss of heterozygosity (LOH) study at the BRCA1 locus is possible. Traditionally, tumor LOH has been studied with the help of microsatellite markers. Unfortunately, this method does not permit discrimination of whether the unclassified mutant allele has been the one selectively retained in the tumor DNA. Hence, additional sequencing analysis is needed to address this issue.

We have developed an analysis based on PCR-denaturing gradient gel electrophoresis (DGGE) that permits us to demonstrate the selective retention of deleterious BRCA1 alleles in DNA extracted from either fresh or paraffin-embedded breast/ovarian tumor tissues.

The extraction protocol for paraffin-embedded tissue DNA was modified from Sarkar et al. (17). Basically, two 10-mm sections from a block of paraffin-embedded tissue were placed in a 1.5-mL microcentrifuge tube after excess paraffin was removed with a scalpel. To dissolve the paraffin, the sections were immersed in 1 mL of xylene.

A cutoff value of 100 ng/L for the ascitic fluid IL-8 concentration yielded 100% sensitivity and 100% specificity for diagnosis of spontaneous bacterial peritonitis in cirrhotic patients.

These data are the preliminary results of a larger study that is still ongoing to establish the possible prognostic value of ascitic fluid IL-8 in SBP patients. Two of 11 SBP patients developed septic shock, both patients having the highest ascitic fluid IL-8 concentrations. Up to now, we conclude that (a) IL-8 concentrations in ascitic fluid in SBP-cirrhotic patients are significantly greater than in patients with SA; (b) ascitic fluid IL-8 concentrations decrease from baseline to 48 h after the initiation of treatment in SBP patients; (c) IL-8 concentrations in ascitic fluid are higher than plasma IL-8 concentrations, suggesting that IL-8 is a local peritoneal production of IL-8 during SBP; (d) IL-8 correctly classified SBP in cirrhotic patients; and (e) IL-8 concentration yielded 100% sensitivity and 100% specificity for diagnosis of spontaneous bacterial peritonitis in cirrhotic patients.

**References**

gently mixed, and incubated for 15 min at room temperature. The tube was then centrifuged for 15 min at 13 000g. The liquid was then removed, and the entire procedure was repeated once. The tissue was rehydrated by repeating the above procedure, first with 1 mL of ethanol and finally with 1 mL of 700 mL/L ethanol. After complete ethanol evaporation, 500 μL of lysis buffer (10 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCl, 2.5 mmol/L MgCl₂, 4.5 mL/L Tween® 20, 100 mg of proteinase K) was added, followed by incubation at 55 °C for 2 h and then at 48 °C until the tissue was completely degraded (4 days at 48 °C, with 50 mg of proteinase K added the second day). Samples were boiled for 10 min and centrifuged at 10 000 g for 10 min. The liquid was carefully removed, and the proteins were extracted with standard phenol/chloroform procedures. The DNA was then precipitated by the addition of a 1:10 volume of 3 mol/L sodium acetate and 2.5 volumes of ethanol (50 μL of sodium acetate and 1.375 μL of ethanol to a 500-μL sample).

Peripheral blood lymphocytes and fresh tissue DNA were extracted according to standard protocols. PCR and DGGE conditions were as follow for all tested BRCA1 exons. A 100-ng sample of control or tumor DNA (Cetus-Perkin-Elmer) in a final volume of 25 μL of the following solution: 200 μmol/L each dNTP (Promega), 1.5 mmol/L MgCl₂, 50 mL/L deionized formamide (Sigma), 0.166 mmol/L (NH₄)SO₄, 67 mmol/L Tris-HCl, pH 8.8, 0.1 mL/L Tween-20. PCR reactions were carried out in a DNA Thermal Cycler PTC 100 (MJ Research). After denaturation at 95 °C for 5 min, 10 cycles at 94 °C for 40 s, 43 °C for 60 s (−0.5 °C per cycle), and 72 °C for 90 s plus 30 cycles at 94 °C for 40 s, 40 °C for 60 s, and 72 °C for 90 s (1 s added per cycle) were performed, followed by a final extension step of 10 min at 72 °C. Each PCR amplification was terminated with a round of heteroduplexing: 98 °C for 10 min, 58 °C for 30 min, and finally 37 °C for 30 min. DGGE analysis was performed in a DGGE System (DGGE-2000; C.B.S. Scientific). A 6-μL aliquot of control or tumor PCR product was mixed with 2 μL of standard dye loading buffer and electrophoresed through a 20-cm 10% acrylamide/bis-acrylamide (37:5:1) gel (20–80% urea-formamide chemical gradient) in 1 × Tris-acetate-EDTA (40 mmol/L Tris, 20 mmol/L sodium acetate, 1 mmol/L EDTA, pH 8) for 12 h at 100V and 58 °C. The gel was stained in a solution of ethidium bromide, and the DNA was photographed under ultraviolet light. The DGGE BRCA1 oligonucleotide primer sequences are available upon request.

A typical DGGE analysis of different BRCA1 exons amplified from both tumor DNA (lane T) and peripheral blood lymphocytes DNA (lane N) is shown in Fig 1. Examples of cancer-predisposing BRCA1 germ-line mutations (11) identified in three families with breast/ovarian cancer syndrome studied in our hospital are shown in Fig. 1A. Four bands corresponding to wild-type and mutant alleles plus two heteroduplex hybrids are apparent for the 589delCT and 5242 C→A cases. Two bands correspond-
fied from paraffin-embedded breast tumor tissues except for the 3238G—A variant, which was amplified from fresh breast tumor tissue.

As far as we know, we report here for the first time the use of DGGE to detect selective retention of deleterious BRCA1 alleles in tumor DNA extracted from either paraffin-embedded or fresh tissues. DNA extracted from paraffin-embedded tissue can be a poor PCR template because it very often is severely damaged, and DGGE primers add difficulties to the PCR reaction because of the long 40- to 50-bp GC-clamps used to improve melting profiles (18). Nonetheless, the method reported here has been applied successfully to the analysis of different BRCA1 exons amplified from several distinct paraffin-embedded tissues.

The present method takes advantage of the fact that BRCA1 is a highly polymorphic gene and that nearly all screened individuals are heterozygous for one of the well-known more common polymorphic sequences (19). The method can be an advantageous alternative to microsatellite studies when analyzing BRCA1 LOH, especially for laboratories that use a DGGE method for screening BRCA1 mutations. However, a comparative study with the standard microsatellite-based LOH assays should be performed.

Analysis of selective allele tumor retention performed in common polymorphic BRCA1 sequences and mutant exons can be a powerful method of defining BRCA1 mutation-associated haplotypes. This method can also be applied to the identification of sporadic breast/ovarian tumor development in women already identified as harboring a germ-line BRCA1 mutation. We also believe that this approach can be applied to the detection of tumor-selective retention of deleterious mutant alleles of tumor suppressor genes other than BRCA1.

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References


Citrate-Theophylline-Adenosine-Dipyridamol Buffer Is Preferable to Citrate Buffer as an Anticoagulant for Flow Cytometric Measurement of Platelet Activation

Margitta Neufeld,1 Ulrike Nowak-Göttli1 and Ralf Junker2

1 Pediatric Hematology and Oncology and 2 Institute of Clinical Chemistry and Laboratory Medicine and Institute of Arteriosclerosis Research, University of Münster, Albert Schweitzer-Strasse 33, 48129 Münster, Germany; * address correspondence to this author at: Institute of Clinical Chemistry and Laboratory Medicine, University of Münster, Albert Schweitzer-Strasse 33, 48129 Münster, Germany; fax 49-251-8347227, e-mail junkerr@uni-muenster.de

A simple, rapid method is needed for collection of platelets for flow cytometric measurement of platelet activation in investigations relating to coronary heart disease, stroke, and peripheral arterial disease (1–6). Specimen collection and sample preparation must minimize activation of platelets (7–9).

The most frequently used anticoagulant for platelet analysis is sodium citrate, but it is deficient because of the difficulty in controlling osmolarity in functional assays (10). Other anticoagulants (EDTA and recombinant thrombin inhibitors such hirudin or low-molecular weight heparin) offer no alternative because of possible interactions with other substances used for analysis (10). In addition, platelets stimulated with ADP in citrate blood usually aggregate if stirred. To prevent clotting and cell-to-cell-adhesion, blood must be diluted and stirring reduced (7) when unfixed platelets are used. However,