<0.001). Wilcoxon analysis, which was used to evaluate the effects of therapy on IL-8 concentrations, showed a statistically significant decrease (P < 0.05) in ascitic fluid IL-8 concentrations after 48 h of therapy. Ascitic fluid IL-8 concentrations on admission and at 48 h after the initiation of treatment are represented in Fig. 1.

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) in SBP patients, IL-8 concentrations in ascitic fluid are higher than plasma IL-8 concentrations, suggesting a local peritoneal production of IL-8 during SBP; (d) IL-8 seems to play a role in SBP in cirrhotic patients; and (e) IL-8 correctly classified SBP and SA patients.

**References**

Denaturing Gradient Gel Electrophoresis-based Analysis of Loss of Heterozygosity Distinguishes Nonobvious, Deleterious **BRCA1** Variants from Nonpathogenic Polymorphisms, Miguel de la Hoya,1,2 Eduardo Díaz-Rubio,1,3 and Trinidad Caldés1,2* (1 Laboratory of Molecular Oncology and Departments of 2 Immunology and 3 Oncology, San Carlos University Hospital, 28040 Madrid, Spain; * address correspondence to this author at: Laboratorio de Oncología Molecular, Planta Baja Sur, Hospital Clínico San Carlos, c/Martín Lagos s/n, 28040 Madrid, Spain; fax 34-1-3303544, e-mail uinvest2@hcsc.es)

**BRCA1** is a tumor suppressor gene (1) responsible for one-half of familial breast/ovarian cancer syndromes and 40% of breast-only cancer syndromes (2, 3). **BRCA1** codes for a 220-kDa nuclear phosphoprotein that has been suggested to play a role in cellular processes, including DNA repair and recombination (4, 5), transcriptional regulation (6, 7), and appropriate chromosomal segregation (8). It is unclear which of **BRCA1** functions are important for decreasing breast/ovarian cancer susceptibility.

**BRCA1** testing and genetic counseling services are offered to families with histories of breast and/or ovarian cancer (9). 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 (10).

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 (11). 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 (12) or disturb the RING-finger domain structure (13) have been defined as cancer-predisposing mutations (11). 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 (10). **BRCA1** somatic mutations have not been detected in sporadic breast cancer (15) and are very uncommon in sporadic ovarian cancer (16). 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,
2.5 volumes of ethanol (50% addition of a 1:10 volume of 3 mol/L sodium acetate and form procedures. The DNA was then precipitated by the proteins were extracted with standard phenol/chloroform procedures. The DNA was then precipitated by the 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.

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 was amplified in the presence of 0.4 μmol/L each oligonucleotide primer and 1 U of Taq polymerase (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 1X 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 listed in Table 1. 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 1A. 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-

gently mixed, and incubated for 15 min at room temperature. The tube was then centrifuged for 15 min at 13 800g. 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.

Signals from both alleles are of identical strength in normal tissue (lane N). In contrast, the mutant allele has been selectively retained in the tumor DNA (lane T), confirming the tumor suppressor role of BRCA1 in those tumors and indicating that any other true deleterious BRCA1 variant will be easily detected with this procedure.

Examples of direct applications of this analytical procedure are shown in Fig. 1B. The 3238G→A mutation is a BRCA1 mutation recorded in the Breast Cancer Informative Core Database as an unclassified variant (11). The experiment clearly shows that both BRCA1 alleles are equally retained in the tumor, indicating that 3238 G→A (which produces a nonconservative S1040N amino acid change in the BRCA1 protein) is not a deleterious BRCA1 mutation and must be considered a nonpathogenic rare polymorphism. This is invaluable information for genetic counseling.

IVS7-34C→T is a common BRCA1 polymorphism (11). When analyzed in a woman harboring a deleterious BRCA1 mutation (the same woman who demonstrates the 5242C→A mutation in Fig. 1A), it is obvious that allele IVS7-34C has been selectively retained in the tumor DNA, indicating that both IVS7-34C and 5242A lie in the same allele. Making the same analysis with other common BRCA1 polymorphisms, we can easily determine BRCA1 mutation-associated haplotypes. Tumor DNA was ampli-

![Fig. 1. DGGE analysis of different BRCA1 exons amplified from both tumor DNA (lane T) and peripheral blood lymphocytes DNA (lane N). (A), analysis from three women harboring BRCA1 germ-line deleterious mutations: 589delCT (exon 8), 1370insATCT (exon 12), and 5242C→A (exon 18). (B), analysis from two women harboring the common BRCA1 polymorphism IVS7-34C→T and the unclassified BRCA1 variant 3238G→A (exon 11). Bands corresponding to wild-type (Wt) and mutant (Mut) alleles are indicated in all cases.](https://academic.oup.com/clinchem/article/45/11/2029/5643397)
eled 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-Adenine-Dipyridamol Buffer Is Preferable to Citrate Buffer as an Anticoagulant for Flow Cytometric Measurement of Platelet Activation
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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 using platelets are used. However,