High-Throughput Mutation Detection Method to Scan BRCA1 and BRCA2 Based on Heteroduplex Analysis by Capillary Array Electrophoresis

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Background: Scanning for mutations in BRCA1 and BRCA2 in a large number of samples is hampered by the large sizes of these genes and the scattering of mutations throughout their coding sequences. Automated capillary electrophoresis has been shown to be a powerful system to detect mutations by either single-strand conformation polymorphism or heteroduplex analysis (HA).

Methods: We investigated the adaptation of gel-based HA of BRCA1 and BRCA2 to a fluorescent multicapillary platform to increase the throughput of this technique. We combined multiplex PCR, three different fluorescent labels, and HA in a 16-capillary DNA sequencer and tested 57 DNA sequence variants (11 insertions/deletions and 46 single-nucleotide changes) of BRCA1 and BRCA2.

Results: We detected all 57 DNA changes in a blinded assay, and 2 additional single-nucleotide substitutions (1186 A>G of BRCA1 and 3624 A>G of BRCA2), previously unresolved by conformation-sensitive gel electrophoresis. Furthermore, different DNA changes in the same PCR fragment could be distinguished by their peak patterns.

Conclusions: Capillary-based HA is a fast, efficient, and sensitive method that considerably reduces the amount of “hands-on” time for each sample. By this approach, the entire coding regions of BRCA1 and BRCA2 from two breast cancer patients can be scanned in a single run of 90 min.

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Mutations in BRCA1 (MIM 113705) and BRCA2 (MIM 600185) account for ~20% of families with evidence of inherited susceptibility to breast cancer (1). The BRCA1 gene is distributed in 24 exons with 5589 nucleotides of coding sequence, which produce a protein of 1863 amino acids (2). The BRCA2 gene is composed of 27 exons and encodes an 11 385-bp transcript that codes for a protein of 3418 amino acids (3).

Genetic testing of BRCA1 and BRCA2 allows identification of mutation carriers who may benefit from tailored screening and prevention protocols. To date, >2600 distinct DNA sequence variants have been described throughout the sequences of BRCA1 and BRCA2 in the BIC database (4). The search for mutations therefore requires extensive analysis of the entire coding sequence of both genes.

Numerous techniques have been developed for the detection of subtle DNA sequence changes, including single-strand conformation polymorphism analysis (5), denaturing gradient gel electrophoresis (6), denaturing HPLC (7), and heteroduplex analysis (HA) (8). All of these techniques have limitations, and many are not suitable for high-throughput strategies, e.g., denaturing gradient gel electrophoresis. HA by conformation-sensitive gel electrophoresis (CSGE) (8) is considered to detect almost 100% of sequence variants and is suitable for high-throughput designs. It has been used extensively in
mutation scanning of many disease-causing genes, including BRCA1 and BRCA2 (9–12). Using this method we have detected 87 different DNA changes in BRCA1 and BRCA2 in ~300 samples from breast cancer patients [Refs. (12, 13) and Infante et al., manuscript in preparation]. However, standard slab gel CSGE is time-consuming and labor-intensive.

The introduction of fluorescent labels as well as gel-based laser detection electrophoresis platforms dramatically improved the throughput of CSGE (14–16), but these types of sequencers are still labor-intensive. Capillary systems substantially reduce the amount of "hands-on" time required to process each sample because long and laborious steps, such as gel preparation and sample loading, are omitted. Several reports have shown that it is possible to transfer fluorescent HA from a gel-based system to a monocapillary system (17–19). These reports demonstrated that capillary HA is fast, sensitive, and reproducible.

With a view to designing a high-throughput method, we adapted our gel-based HA of BRCA1 and BRCA2 to a fluorescent multicapillary system. We tested the ability of this method to detect 57 DNA changes, 11 insertions/deletions and 46 single-nucleotide substitutions (SNS; 25 in BRCA1 and 32 in BRCA2), previously detected by standard CSGE.

Materials and Methods

DNA ISOlATION AND SAMPLES

DNA from breast cancer patients and controls was extracted from EDTA-anticoagulated blood samples by standard protocols. The 57 DNA changes investigated comprised 46 SNS and 11 small insertions or deletions, including 10 frameshift mutations and 1 intronic insertion (Tables 1 and 2). Mutation nomenclature was basically according to den Dunnen and Antonarakis (20).

MULtiplex PCR

Exons 2–24 of BRCA1 and 2–27 of BRCA2 were amplified in 72 PCR fragments [30 for BRCA1 and 42 for BRCA2; primer sequences were kindly provided by M. Stratton (Institute of Cancer Research, Sutton, Surrey, UK), and are shown in Tables 3 and 4 of the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol50/issue2/]. Some primer pairs were newly designed (Tables 3 and 4 in the online Data Supplement). Fragments ranged in size from 179 to 584 bp (BRCA1) and from 194 to 575 bp (BRCA2). Large exons, such as BRCA1 exon 11 and BRCA2 exons 10, 11, 14, and 27, were amplified in several overlapping fragments.

These fragments were divided into 23 multiplex-PCR groups in relation to their sizes and compatible amplification conditions (Tables 3 and 4 in the online Data Supplement). One primer of each pair was labeled with 6-carboxyfluorescein (FAM), hexachloro-6-carboxyfluorescein (HEX), or 2′,7′-benzo-5′-fluoro-2′,4′,7′-trichloro-5-carboxyfluorescein (NED; Dye Set D; Applied Biosystems). Multiplex PCRs for large fragments were carefully set up to ensure optimum amplification of these fragments and to obtain peak values similar to those for other, smaller fragments in the same reaction. As a general rule in each multiplex group, larger fragments required higher concentrations of primers.

PCRs were carried out in a final volume of 25 μL containing 200 ng of genomic DNA; primers at the concentrations specified in Tables 3 and 4 of the online Data Supplement; 600 μM each deoxynucleotide triphosphate; AmpliTaq Gold 1× buffer (Applied Biosystems) or OptiPrime Buffer #6 (Stratagene), as indicated in Tables 3 and 4 of the online Data Supplement; MgCl2 at 2.0–2.5 mM when using AmpliTaq Gold buffer; and 1 U of AmpliTaq Gold or 2.5 U of AmpliTaq DNA polymerase (Applied Biosystems).

BRCA1 exon 7 could not be included in this analysis because three very frequent insertion/deletion polymorphisms [6–7 copies of the trinucleotide CTT and 1 or 2 copies of C(T10–12)] are located 17 and 40 bp downstream of the 3′ end of exon 7. Consequently, most samples show

<table>
<thead>
<tr>
<th>Table 1. BRCA1 DNA changes analyzed by capillary electrophoresis.</th>
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<tbody>
<tr>
<td>DNA change* (amino acid change)</td>
</tr>
<tr>
<td>IVS1-115 T&gt;C</td>
</tr>
<tr>
<td>IVS2-14 C&gt;T</td>
</tr>
<tr>
<td>233 G&gt;A</td>
</tr>
<tr>
<td>330 A&gt;G</td>
</tr>
<tr>
<td>IVS5 + 1 G&gt;A</td>
</tr>
<tr>
<td>655 A&gt;G (Y179C)</td>
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<tr>
<td>IVS8-58delT</td>
</tr>
<tr>
<td>710 C&gt;T</td>
</tr>
<tr>
<td>1135insA</td>
</tr>
<tr>
<td>2201C&gt;T</td>
</tr>
<tr>
<td>2731 C&gt;T (P871L)</td>
</tr>
<tr>
<td>3232 A&gt;G (E1038G)</td>
</tr>
<tr>
<td>3667 A&gt;G (K1183R)</td>
</tr>
<tr>
<td>3958delCTCAG;insAGGC</td>
</tr>
<tr>
<td>IVS11 + 15 T&gt;C</td>
</tr>
<tr>
<td>4427 T&gt;C</td>
</tr>
<tr>
<td>IVS13-10 C&gt;T</td>
</tr>
<tr>
<td>IVS14-63 C&gt;G</td>
</tr>
<tr>
<td>4654 G&gt;T (S1512I)</td>
</tr>
<tr>
<td>4956 A&gt;G (S1613G)</td>
</tr>
<tr>
<td>5236 G&gt;A (G1706E)</td>
</tr>
<tr>
<td>5242 C&gt;A (A1708E)</td>
</tr>
<tr>
<td>IVS18-1 G&gt;A</td>
</tr>
<tr>
<td>IVS20 + 1 G&gt;A</td>
</tr>
<tr>
<td>IVS19-2 A&gt;G</td>
</tr>
</tbody>
</table>

* Mutations studied to establish optimal HA conditions are shown in bold. Disease-causing mutations are shown in italics. Amino acid changes are shown only for missense and nonsense mutations. 

UV, unclassified variant; P, polymorphism; Sp, splicing; M, missense; D, deletion; I, insertion; Fr, frameshift; N, nonsense.
Two different sets of thermocycling conditions were performed in a GeneAmp PCR System 9700 (Applied Biosystems; see Tables 3 and 4 in the online Data Supplement): For BRCA1 groups, thermocycling included 35 cycles of 94 °C for 1 min, 56 °C for 1 min, and 72 °C for 2 min. For BRCA2 groups, thermocycling included the following touchdown protocol (16): 35 cycles of 94 °C for 45 s, and touchdown annealing temperatures from 60 to 50 °C for 45 s and 72 °C for 60 s. However, to avoid nonspecific bands from old DNA samples, we also amplified the groups 2D, 2F, 2I, and 2K with the following conditions: 35 cycles of 94 °C for 45 s, 52 °C for 45 s, and 72 °C for 60 s.

An initial denaturation step at 95 °C for 2 min (10 min when using AmpliTaq Gold) and a final extension step at 72 °C for 5 min were added to all thermocycling protocols.

### HA

A heteroduplex formation step was performed in the same PCR instrument and consisted of heating the PCR products to 98 °C for 5 min and allowing them to cool to 25 °C in six steps: cooling to 90 °C over 5 min, cooling to 80 °C over 5 min, cooling to 75 °C over 10 min, cooling to 60 °C over 10 min, cooling to 40 °C over 20 min, and cooling to 25 °C over 20 min.

HA by manual CSGE was performed according to previously reported protocols (8).

Fluorescent HA was carried out under nondenaturing conditions as follows. PCR products labeled with FAM, HEX, and NED were pooled together and diluted 1:5–1:20 in deionized water with 0.5 μL of Genescan 500-ROX or Genescan 2500-ROX size calibrators (Applied Biosystems). These size calibrators were used to compensate for any inter- and intracapillary variations in electrophoretic migration.

All PCR products were resolved on an ABI 3100 DNA sequencer (16 capillaries; Applied Biosystems). This instrument has a capacity of two plates of 96 or 384 samples, which are injected automatically during the run. The matrix concentration was varied between 3% and 5% Genescan polymer (Applied Biosystems) according to a previous report (17), with 0–100 mL/L glycerol, and 1× Tris-borate-EDTA (TBE) or 1× electrophoresis buffer with EDTA (Applied Biosystems). Denaturing agents, such as formamide or urea, were omitted because they reduce the lifespan of the capillary and do not significantly improve the resolution of heteroduplexes (17). The capillary arrays were 36 and 50 cm long (Applied Biosystems). Run temperatures (20–35 °C), run voltages (5, 7.5, 10, 12.5, and 15 kV), sample injection voltages and times, and run times (1.5 h) were selected in the Data Collection Software, Ver. 1.1 (Applied Biosystems).

Fragment analysis was carried out blinded by use of Genescan software, Ver. 3.7 (Applied Biosystems). Heteroduplexes were identified by the presence of altered peak patterns compared with a control sample.

### SEQUENCING

Fragments showing an altered pattern were sequenced with the BigDye® Terminator Sequencing Kit, Ver. 3.1 (Applied Biosystems), with unlabeled forward and reverse primers.
Results
Optimization of electrophoretic conditions
To determine the best resolution settings, we evaluated different run conditions with use of 15 randomly selected DNA sequence variants (11 SNS and 4 insertions/deletions). These mutations are shown in bold font in Tables 1 and 2. Multiplex PCRs were performed as described in the Materials and Methods.

We assessed capillary length (36 and 50 cm), run voltage, run temperature, different concentrations of Genescan polymer (3–5%) and glycerol (0–100 mL/L), and two electrophoresis buffers, 1× TBE and 1× ABI, both with 0–100 mL/L glycerol.

A polymer concentration of 5% and the presence of glycerol (100 mL/L) improved the resolution of all DNA changes. In addition, intermediate run voltages (10–12.5 kV) and low temperatures (20–25 °C) favored the detection of the DNA variants. However, with regard to the resolution of heteroduplexes, no clear differences were observed when we used run voltages of 10–12.5 kV and temperatures of 20–25 °C. Nevertheless, visualization of the otherwise poorly resolved heteroduplexes showed certain benefits at 10 kV and 20 °C (data not shown). The 50-cm capillary array, which was in principle suitable just for sequencing, gave much better resolution of peak shifts than the 36-cm array, which did not distinguish wild-type from mutant samples for several SNS.

The final, optimum electrophoretic conditions were as follows: 5% Genescan polymer in 1× TBE buffer with 100 mL/L glycerol, 1× TBE with 100 mL/L glycerol as electrophoresis buffer, a 50-cm capillary array, 10 kV, and 20 °C for 90 min. Under these conditions, anomalous patterns for all 15 DNA variants were detected. Any changes in these conditions led to poor resolution of heteroduplexes or no detection of the mutations. For example, the choice of other electrophoresis buffers, such as 1× ABI with or without glycerol or 1× TBE without glycerol, prevented the detection of at least 1 of these 15 DNA changes. The dramatic effects of the electrophoresis buffer and glycerol on peak patterns are illustrated in Fig. 1. The mutation IVS20 + 1 G>A at exon 20 of BRCA1 clearly showed four different peak patterns, which went from a pattern showing two overlapping peaks (1× ABI buffer +100 mL/L glycerol; Fig. 1B) to the best resolution, with a pattern of four peaks (1× TBE + 100 mL/L glycerol; Fig. 1D) corresponding to the two homoduplexes and the two heteroduplexes.

Identification of altered peak patterns in 59 DNA changes
We had previously identified 87 different DNA changes by CSGE (data not shown). To compare the sensitivity of standard CSGE with that of HA in a fluorescent multi-capillary system, we evaluated 57 BRCA mutants (25 in BRCA1 and 32 in BRCA2), including the 15 previously analyzed mutations. Because single-base changes are the most commonly occurring mutations in genetic diseases, the sensitivity of any mutational scanning technique is determined by its ability to detect these types of changes. For this reason, we studied 46 SNS, of which 18 were

![Fig. 1. Mutation IVS20 + 1 G>A of BRCA1 FAM-exon 20 assayed with different buffer compositions.](A) 1× ABI buffer; (B) 1× ABI buffer + 100 mL/L glycerol; (C) 1× TBE; (D) 1× TBE + 100 mL/L glycerol. The four experiments were performed under the following fixed conditions: 5% Genescan polymer with either 1× ABI or 1× TBE buffer and with or without 100 mL/L glycerol; 50-cm capillary length; 10 kV at 20 °C for 90 min.)
intronic or exonic variants, 18 were missense, 5 were nonsense, and 5 were splicing mutations. The remaining 11 alterations were small insertions or deletions (1 intronic and 10 frameshift mutations).

All of the samples carrying these DNA changes, as well as the control samples, were processed as described in the Material and Methods. All of the insertions/deletions showed a clearly altered peak pattern, which in general contained the four expected bands corresponding to the two homoduplexes and the two heteroduplexes (Fig. 2A).

The altered peak patterns of SNS were quite variable and complex. Basically, four different patterns could be distinguished: one abnormal peak (Fig. 2B), two peaks (Fig. 2C), three peaks (Fig. 2D), and four peaks that corresponded to the two homoduplexes and the two heteroduplexes (Fig. 2E). The most difficult pattern to identify was the first one (one abnormal peak), but it was also the least common type. Other patterns with two, three, or four overlapping peaks could also be identified.

All 57 DNA variants were detected under the conditions described above. Moreover, peak shifts corresponding to two additional single-nucleotide changes, which we had not detected previously by CSGE, were also visualized when we tested multiplex groups of one patient carrying IVS20 + 1G>A (group 1A in Table 3 of the online Data Supplement) and one control sample (group 2J in Table 4 of the online Data Supplement). These mutations were as follows: 1186 A>G (BRCA1 exon 11a2; BIC no. 1014) and 3624 A>G (BRCA2 exon 11GH; BIC no. 1661). Both were in large fragments of 453 and 566 bp, respectively. Remarkably, 12 of 59 mutations (20%) detected by capillary HA were located within fragments >500 bp, considered as the upper limit for CSGE, and all of them were successfully identified (data not shown).

SPECIFICITY OF CAPILLARY HA
To establish the specificity of the capillary HA method, we compared patterns from different mutations in the

![Fig. 2. Peak patterns of insertions/deletions and SNS.](image)

(A), frameshift mutation 1538delAAGA from exon fragment FAM-10BD1 of BRCA2; (B), 3199G>A from exon fragment FAM-11EF of BRCA2; (C), 3667 A>G from exon fragment NED-11d2 of BRCA1; (D), 7288 C>T from HEX-exon 14-A of BRCA2; (E), IVS5 + 1 G>A from FAM-exon 5 of BRCA1.
same PCR fragment (see Tables 1 and 2) under the optimum settings. All of the mutations in each amplicon could be distinguished by their patterns. Shown in Fig. 3A are the patterns for the mutations 3690 C>T, 3624 A>G, and 3492insT from BRCA2 exon fragment 11GH. In addition, wild-type homozygous samples could be differentiated from mutant homozygous samples for some frequent polymorphisms, such as 3667 A>G (K1183R) of BRCA1 exon fragment 11d2, which was identified in >30% of samples and has been classified as a missense polymorphism (BIC database) (4). Shown in Fig. 3B are the three expected patterns for mutation K1183R: homozygous wild type (wt/wt), heterozygous wt/K1183R, and homozygous K1183R/K1183R.

**Discussion**

The purpose of this study was to develop a sufficiently sensitive, high-throughput method to scan the entire coding sequences of BRCA1 and BRCA2 with a view to offering rapid and reliable genetic analysis to breast and ovarian cancer patients. This new method may be of interest for genetic testing laboratories that routinely screen BRCA1 and BRCA2 in a large number of samples.

Preliminary studies had demonstrated increased throughput of HA and single-strand conformation polymorphism analysis by the inclusion of fluorescent labels and gel electrophoresis in automated sequencers (14–16). Additional progress in throughput was achieved when electrophoresis was transferred to capillary systems (17, 21). It was suggested that further advances in throughput could be achieved by use of multicapillary systems and different labels.

We report here substantial improvement in the HA of BRCA1 and BRCA2 by use of fluorescent 16-capillary platforms that provide a faster, high-throughput, reproducible, and more sensitive method. The increase in the throughput of our method relies on four features: (a) use of a 16-capillary system, which reduces to a large extent the time needed to process each sample; (b) multiplex PCR, which allows a decrease in the number of PCR reactions/sample [BRCA1 and BRCA2 can be amplified in 72 PCR fragments divided into only 23 multiplex PCR...
groups (see Tables 3 and 4 in the online Data Supplement); (c) use of different labels for each multiplex PCR

group (FAM, HEX, and NED), which allows the mixture of
different labels to be analyzed in a single capillary array.
In contrast, it is difficult to analyze more than one DNA
fragment using the ABI3100 automated sequencer, which
requires at least one plate per sample. The ABI3100 expense
of $10 479.95 corresponds to only 60% of the cost of the
same capillary array. We estimate that the total cost
submitted we had conducted more than 150 assays with
the 16 capillaries, and at the time this manuscript was
in preparation we had conducted more than 150 assays with
the ABI3100 automated sequencer (26). We therefore
strongly recommend the use of capillary HA as a prescreening
strategy.

Finally, there are other possible ways to increase the
throughput of the technique. One way is through the use of
robotic systems to set up PCR reactions and post-PCR
procedures, which would greatly reduce operation time.
Another way is through the use of 96-capillary platforms,
which would increase the throughput by sixfold. A third
way is through the introduction of five different labels,
which are currently available (Dye Set G5; Applied Bio
systems), and a fourth way is through the development of
specific software that automatically identifies putative
abnormal peak patterns. In fact, we are currently collab-
orating with a bioinformatics group for this purpose.

In conclusion, fluorescent HA of BRCA1 and BRCA2 in
multicapillary automated sequencers is fast, easy, inex-
pensive, and sensitive and allows the mutational analysis
of both genes in a short period of time. This method could
be useful for mutational screening of other large and
complex genes, such as NF1 (MIM 162200), APC (MIM
175100), or P53 (MIM 191170), to speed up their test times.
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