Clinical Chemistry 50:2
313–320 (2004)

Molecular Diagnostics and Genetics

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.**

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)[1] *(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

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Received July 7, 2003; accepted November 24, 2003.

Previously published online at DOI: 10.1373/clinchem.2003.023614

[1] Nonstandard abbreviations: HA, heteroduplex analysis; CSGE, conformation-sensitive gel electrophoresis; SNS, single-nucleotide substitution; FAM, 6-carboxyfluorescein; HEX, hexachloro-6-carboxyfluorescein; NED, 2,7′,8′-benzo-5′-fluoro-2′,4,7-trichloro-5-carboxyfluorescein; and TBE, Tris-borate-EDTA.

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′,8′-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; $MgCl_2$ 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 $C(T_{10-12})$] are located 17 and 40 bp downstream of the 3′ end of exon 7. Consequently, most samples show

Table 1. *BRCA1* DNA changes analyzed by capillary electrophoresis.

DNA change[a] (amino acid change)	Type of change (effect)	Exon (fragment)	BIC number *(4)* or Ref.
IVS1-115 T>C	SNS (UV)[b]	2	Velasco et al. *(13)*
IVS2-14 C>T	SNS (P)	3	2637
233 G>A	SNS (P)	3	1317
330 A>G	SNS (Sp)	5	1944
IVS5 + 1 G>A	SNS (Sp)	I-5	2843
655 A>G (Y179C)	SNS (M)	8	1690
IVS8-58delT	D (P)	I-8	1322
710 C>T	SNS (P)	9	1237
1135insA	I (Fr)	11 (a2)	1008
2201C>T	SNS (P)	11 (e1)	1047
2731 C>T (P871L)	SNS (M)	11 (c)	1067
3232 A>G (E1038G)	SNS (M)	11 (d1)	1087
3667 A>G (K1183R)	SNS (M)	11 (d2)	1099
3958delCTCAG;insAGGC	D-I (N)	11 (g)	4365
IVS11 + 15 T>C	SNS (UV)	11 (g)	Velasco et al. *(13)*
4427 T>C	SNS (P)	13	1128
IVS13-10 C>T	SNS (P)	14	2419
IVS14-63 C>G	SNS (P)	15	4234
4654 G>T (S1512I)	SNS (M)	15	1136
4956 A>G (S1613G)	SNS (M)	16	1140
5236 G>A (G1706E)	SNS (M)	18	3393
5242 C>A (A1708E)	SNS (M)	18	1147
IVS18-1 G>A	SNS (Sp)	19	3942
IVS20 + 1 G>A	SNS (Sp)	20	2100
IVS19-2 A>G	SNS (Sp)	20	3400

[a] 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.

[b] UV, unclassified variant; P, polymorphism; Sp, splicing; M, missense; D, deletion; I, insertion; Fr, frameshift; N, nonsense.

Table 2. *BRCA2* DNA changes analyzed by capillary electrophoresis.

DNA change[a] (amino acid change)	Type of change (effect)	Exon (fragment)	BIC number *(4)* or Ref.
203 G>A	SNS (P)[b]	2	1666
IVS6-19 C>T	SNS (P)	7	2164
IVS8 + 56 C>T	SNS (P)	8	2411
1538delAAGA	D (Fr)	10 (BD1)	2885
1342 C>A (H372N)	SNS (M)	10 (BD1)	1668
1593 A>G	SNS (P)	10 (BD1)	1106
3036delACAA	D (Fr)	11 (EF)	1021
3199 G>A (N991D)	SNS (M)	11 (EF)	1903
3492insT	I (Fr)	11 (GH)	2067
3690 C>T	SNS (UV)	11 (GH)	Velasco et al. *(13)*
4150 G>T (E1308X)	SNS (N)	11 (IL2)	2480
4486 G>T (D1420Y)	SNS (M)	11 (MO)	1273
4898 C>G (T1557S)	SNS (M)	11 (PQ)	4874
5374delTATG	D (Fr)	11 (R)	Duran et al. *(12)*
5972 C>T (T1915M)	SNS (M)	11 (UV)	1108
6126delT	D (Fr)	11 (UV)	Duran et al. *(12)*
6503delTT	D (Fr)	11 (WX)	1047
6518 C>T (T2097M)	SNS (M)	11 (WX)	2297
6884 C>G (S2219X)	SNS (N)	11 (YZ)	1054
7288 C>T (Q2354X)	SNS (N)	14 (A)	Duran et al. *(12)*
7365 A>G	SNS (UV)	14 (A)	Velasco et al. *(13)*
7470 A>G	SNS (P)	14 (B)	1125
IVS14 + 53 C>T	SNS (P)	14 (B)	2738
IVS16-14 T>C	SNS (P)	I-16	1126
9078 G>T (K2950N)	SNS (M)	22	1405
9254delATCAT	D (Fr)	23	1073
9266 C>T (T3013I)	SNS (M)	23	1415
9476 A>T (K3083X)	SNS (N)	24	Duran et al. *(12)*
9520 T>C (Y3098H)	SNS (M)	25	1078
9538delAA	D (Fr)	25	1422
10204 A>T (K3326X)	SNS (N-P)	27 (B)	1179
10462 A>G (I3412V)	SNS (M)	27 (B)	1452

[a] 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.

[b] P, polymorphism; D, deletion; Fr, frameshift; M, missense; I, insertion; UV, unclassified variant; N, nonsense.

band shifts in HA that mask the finding of other exon 7 DNA changes. The proximity of one of these polymorphisms to the exon 7–intron 7 boundary (only 17 bp) forces the design of a reverse primer spanning the 5′ splice site of intron 7; mutations affecting it would therefore be missed. We thus searched for mutations in exon 7 by direct sequencing.

THERMOCYCLING CONDITIONS

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 multicapillary 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

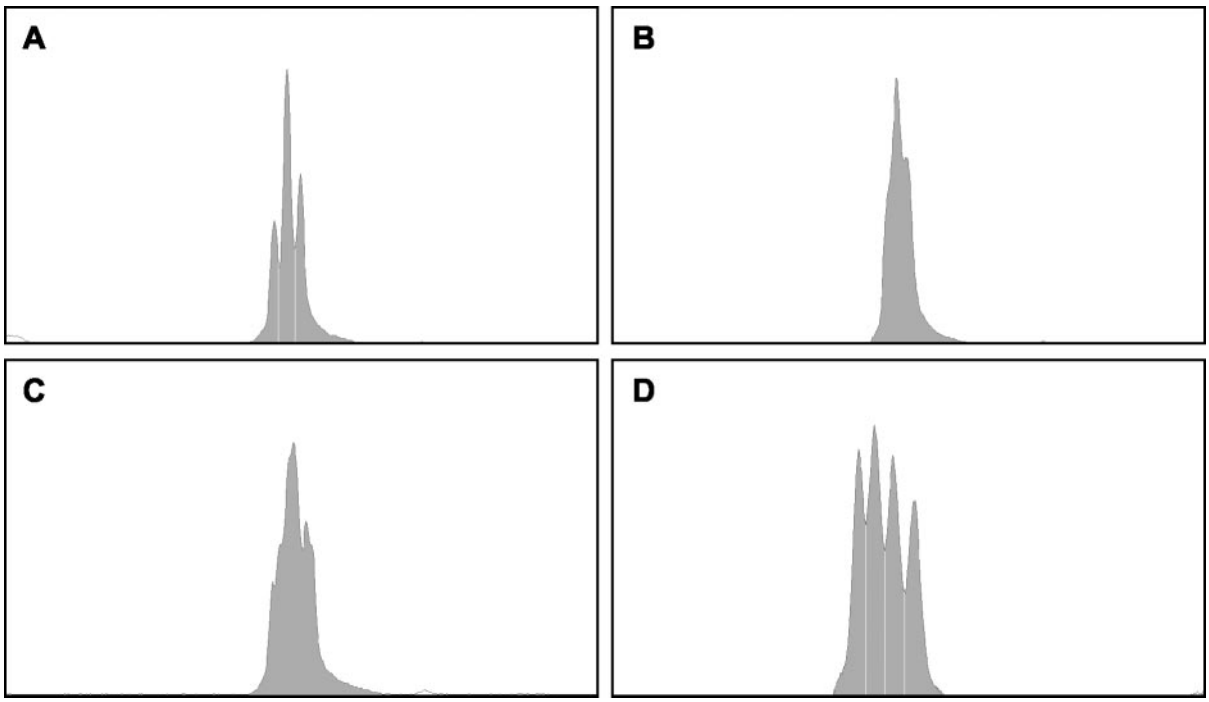

Fig. 1. Mutation IVS20 + 1 G>A of *BRCA1* FAM-exon 20 assayed with different buffer compositions.

(*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

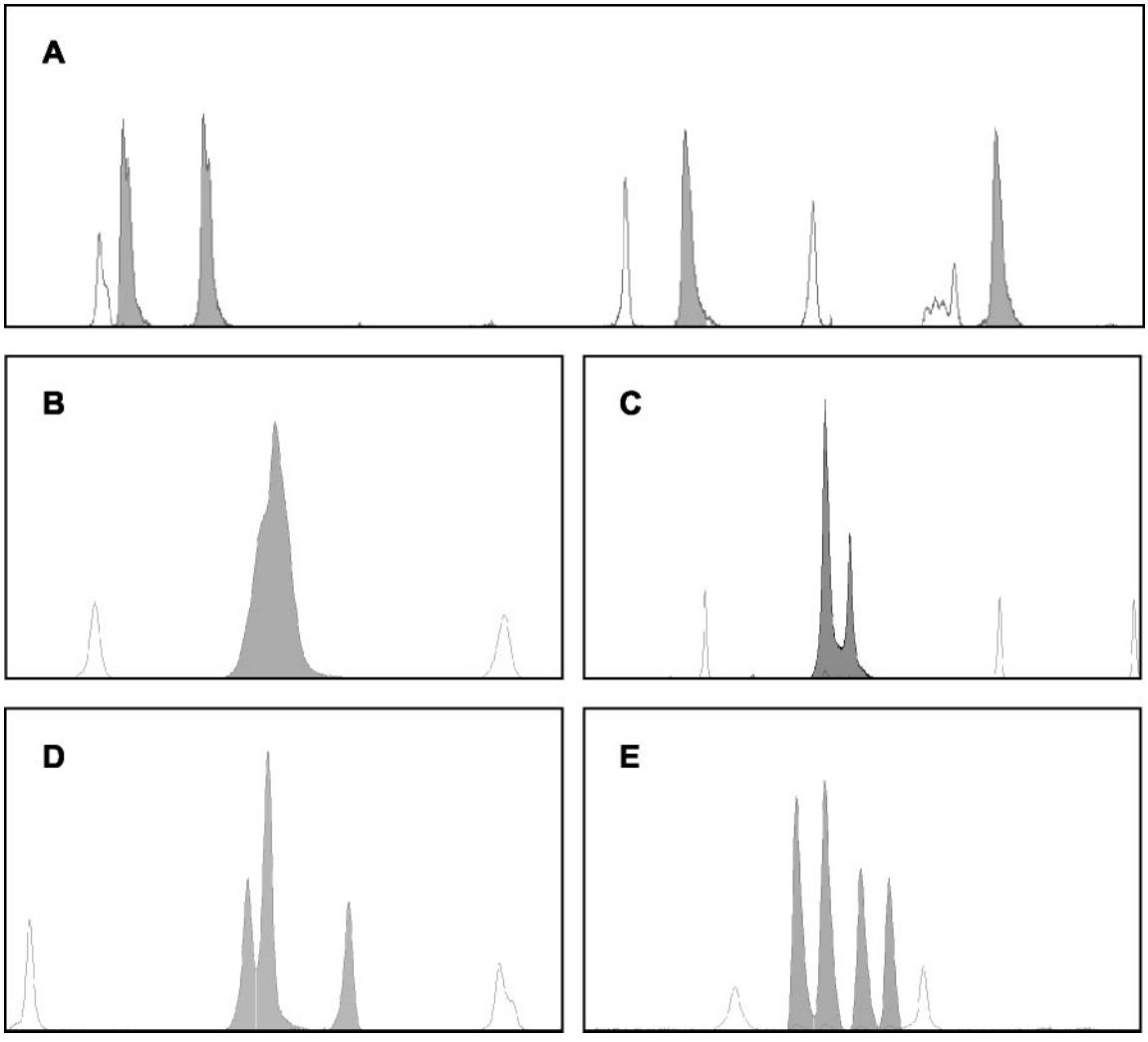

Fig. 2. Peak patterns of insertions/deletions and SNS.

(*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*.

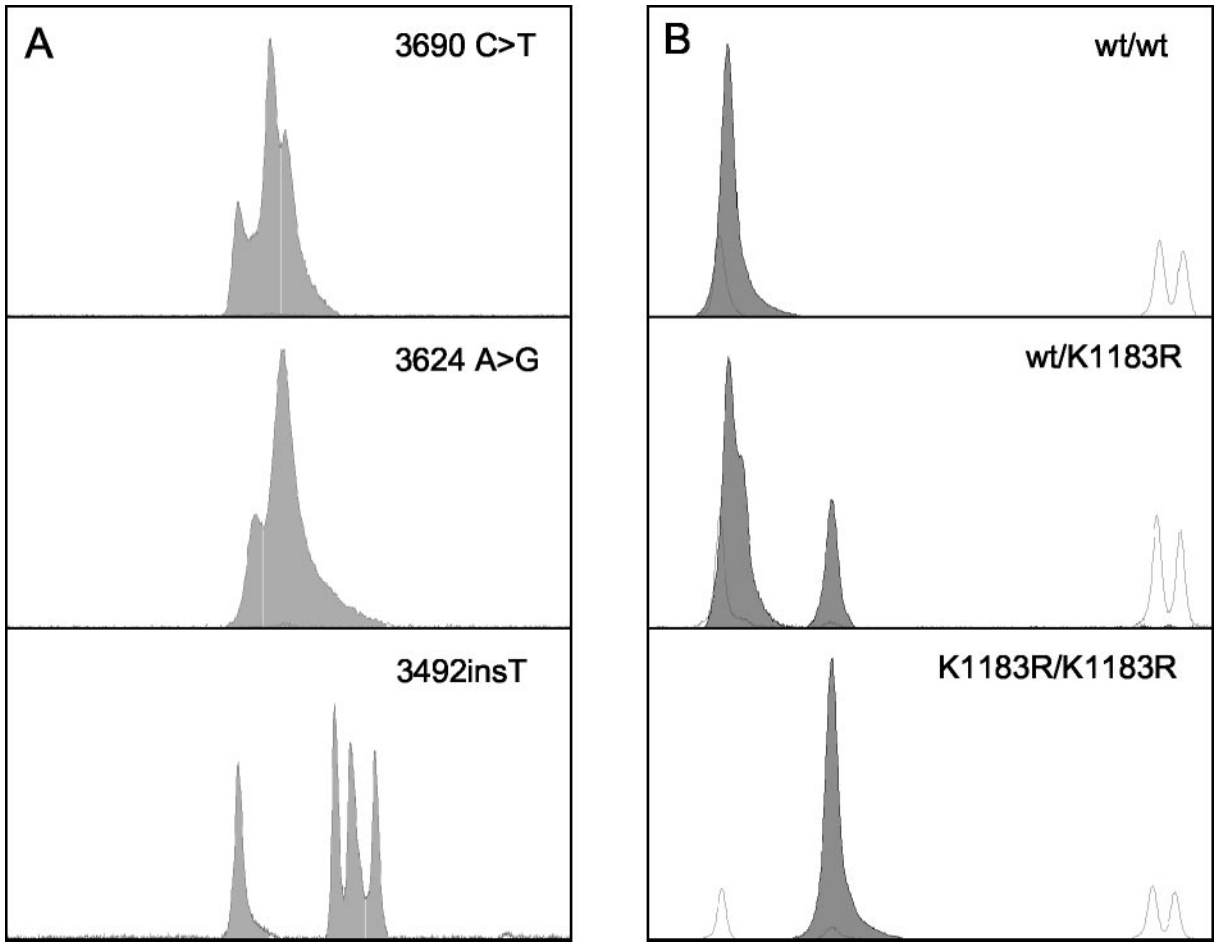

Fig. 3. Electropherograms showing the specificity of capillary HA.

(*A*), peak patterns of three different mutations at the same exon fragment (FAM-ex11GH of *BRCA2*); (*B*), differentiation of homozygous wild-type (*wt/wt*) samples (*top panel*), heterozygous samples for mutation 3667 A>G (*wt/K1183R*; *middle panel*), and homozygous mutant samples (*K1183R/K1183R*; *bottom panel*).

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 three differently labeled multiplex-PCR fragments in one tube; and (*d*) use of a single assay per sample. In contrast, in other capillary-based mutation detection techniques, different sets of conditions have been suggested to ensure detection of all mutations *(19, 22)*. This would considerably increase the amount of data to be analyzed, specially when a large number of samples are to be screened.

Thus, 8–10 PCR fragments can be analyzed in one capillary per run, meaning that analysis of the entire coding regions of *BRCA1* and *BRCA2* from two patients can be performed in a single run of 1.5 h.

Using this method, we have detected all 57 tested mutations. Given that more than 60% of the pathogenic mutations reported in the BIC database are frameshifts *(4)*, it should be pointed out that all insertions/deletions were easily detected by virtue of their peak patterns (Fig. 2A). However, one of the main aims of this study was to confirm the feasibility and sensitivity of our method for the detection of SNS (46 of 57 DNA changes assayed were SNS) because there is increasing evidence implicating SNS in inherited breast cancer through alteration of either the protein function (missense mutations) *(23)* or the RNA splicing as a result of disruption of exonic splicing enhancers (any exonic DNA change, including silent mutations) *(24)*. Remarkably, all of the SNS analyzed were detected with high accuracy. Furthermore, the sensitivity of heteroduplex detection was improved with respect to standard CSGE; capillary HA identified two other mutations (1186A>G of *BRCA1* exon 11a2 and 3624 A>G of *BRCA2* exon 11GH) previously not detected by CSGE. Both mutations are located in two large fragments: 453 bp (11a2) and 566 bp (11GH). The reason is that all fragments must migrate 50 cm from the loading end of the capillary array to the detection window. The increased length of migration allows better resolution of closely migrating bands *(16, 25)*. In standard CSGE, larger fragments may migrate only a short distance (10 cm for fragments of ~500 bp) to retain the shorter fragments for visualization. In fact, band shifts corresponding to these two "new" mutations were also observed in standard CSGE after we increased the electrophoresis run time. According to the BIC database, 11 of the 48 SNS detected had been found only by direct sequencing *(4)*. Altogether these results suggest that our method may have an accuracy similar to that of direct sequencing, regarded as the gold standard technique for mutation detection, although an additional comparative study should be conducted to corroborate this point.

Several authors have reported that the sensitivities of HA and single-strand conformation polymorphism analysis depend on the locations of mutations with respect to the ends of the PCR fragment *(8, 17)*. It has been suggested that mutations located within 50 bp of the ends of the PCR product are difficult to detect. We have not found any effect of a mutation's position on its detection because in our sample 6 of 57 DNA changes were of this type (DNA variants IVS1-115 T>C, IVS8-58delT, and 710 C>T from *BRCA1* and 1342 C>A, 1593 A>G, and 10462 A>G from *BRCA2*).

The different peak patterns of distinct single nucleotide changes in the same fragment support the specificity of this technique (Fig. 3A). Therefore, a specific peak pattern could be assigned automatically to one mutation. Additionally, homozygous wild-type samples can be differentiated from homozygous mutant samples in some frequent DNA variants (Fig. 3B). However, taking into account the presence of slightly altered peak patterns (Fig. 2B), sequencing of all anomalous migration patterns is recommended to differentiate possible false positives.

It is also worth mentioning that the cost per sample of this technique is low. Other than the purchase price of a DNA sequencer and the costs related to the common procedures for both techniques, the total costs per sample for *BRCA1* and *BRCA2* analysis by capillary HA are similar to those of CSGE (in fact, €6, or US $6.90 less in the case of capillary HA). The relatively high costs of the ABI3100 consumables (principally the capillary array and the polymer) are compensated by the minimal consumption of reagents and the long lifespan of the capillary array. In fact, only 50–80 μL of polymer are needed to fill the 16 capillaries, and at the time this manuscript was submitted we had conducted more than 150 assays with the same capillary array. We estimate that the total cost per sample of our technique is approximately eightfold lower than that of direct sequencing, as stated by other authors *(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 Biosystems), and a fourth way is through the development of specific software that automatically identifies putative abnormal peak patterns. In fact, we are currently collaborating with a bioinformatics group for this purpose.

In conclusion, fluorescent HA of *BRCA1* and *BRCA2* in multicapillary automated sequencers is fast, easy, inexpensive, 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.

This work was supported by the Consejería de Sanidad (Junta y Castilla y León) through the regional Breast Cancer Prevention Program. We thank Prof. Michael Stratton for providing most of the primer sequences used in this study, and Dr. Marivi Carretero and Soledad Sánchez del Alamo from Applied Biosystems (Spain) for their technical support. We are also grateful to the patients and the clinicians, particularly Dr. García Girón and his team (Hospital General Yagüe, Burgos, Spain), who collaborated in this study. Finally, we also thank Dr. Eduardo Arranz, Dr. Susana Gómez, and Alan Hynds for critical reading of this manuscript and helpful discussions.

References

1. Wooster R, Weber B. Breast and ovarian cancer [Review]. N Engl J Med 2003;348:2339–47.
2. Miki Y, Swensen J, Shattuck-Eidens D, Futreal PA, Harshman K, Tavtigian K, et al. A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. Science 1994;266:66–71.
3. Tavtigian SV, Simard J, Rommens J, Couch F, Shattuck-Eidens D, Neuhausen S, et al. The complete BRCA2 gene and mutations in chromosome 13q-linked kindreds. Nat Genet 1996;12:333–7.
4. The Breast Cancer Information Core database: http://research.nhgri.nih.gov/bic/Member/index.shtml (Accessed June 2003).
5. Orita M, Iwahana H, Kanazawa H, Hayashi K, Sekiya T. Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. Proc Natl Acad Sci U S A 1989;86:2766–70.
6. Fischer SG, Lerman LS. DNA fragments differing by single base-pair substitutions are separated in denaturing gradient gels: correspondence with melting theory. Proc Natl Acad Sci U S A 1983;80:1579–83.
7. Liu W, Smith DI, Rechtzigel KJ, Thibodeau SN, James CD. Denaturing high performance liquid chromatography (DHPLC) used in the detection of germline and somatic mutations. Nucleic Acids Res 1998;26:1396–400.
8. Ganguly A, Rock MJ, Prockop DJ. Conformation-sensitive gel electrophoresis for rapid detection of single-base differences in double-stranded PCR products and DNA fragments: evidence for solvent-induced bends in DNA heteroduplexes. Proc Natl Acad Sci U S A 1993;90:10325–9.
9. Couch FJ, Farid LM, DeShano ML, Tavtigian SV, Calzone K, Campeau L, et al. BRCA2 germline mutations in male breast cancer cases and breast cancer families. Nat Genet 1996;13:123–5.
10. Markoff A, Sormbroen H, Bogdanova N, Preisler-Adams S, Ganev V, Dworniczak B, et al Comparison of conformation-sensitive gel electrophoresis and single-strand conformation polymorphism analysis for detection of mutations in the BRCA1 gene using optimized conformation analysis protocols. Eur J Hum Genet 1998;6:145–50.
11. Blesa JR, Garcia JA, Ochoa E. Frequency of germ-line BRCA1 mutations among Spanish families from a Mediterranean area [Letter]. Hum Mutat 2000;15:381–2.
12. Duran M, Esteban-Cardenosa E, Velasco E, Infante M, Miner C. Mutational analysis of BRCA2 in Spanish breast cancer patients from Castilla-Leon: identification of four novel truncating mutations [Mutation in Brief]. Hum Mutat 2003;21:448.
13. Velasco Sampedro E, Esteban Cardenosa E, Infante Sanz M, Duran Dominguez M, Lastra Aras E, Garcia Giron C, et al. Molecular study of the BRCA1 and BRCA2 genes in 153 breast cancer families from Castilla and Leon (Spain): new nine unclassified variants identified. Med Clin (Barc) 2002;119:441–5.
14. Ganguly T, Dhulipala R, Godmilow L, Ganguly A. High throughput fluorescence-based conformation-sensitive gel electrophoresis (F-CSGE) identifies six unique BRCA2 mutations and an overall low incidence of BRCA2 mutations in high-risk BRCA1-negative breast cancer families. Hum Genet 1998;102:549–56.
15. Blesa JR, Hernandez-Yago J. Adaptation of conformation-sensitive gel electrophoresis to an ALFexpress DNA sequencer to screen BRCA1 mutations. Biotechniques 2000;28:1019–25.
16. Edwards SM, Kote-Jarai Z, Hamoudi R, Eeles RA. An improved high throughput heteroduplex mutation detection system for screening BRCA2 mutations-fluorescent mutation detection (F-MD). Hum Mutat 2001;17:220–32.
17. Rozycka M, Collins N, Stratton MR, Wooster R. Rapid detection of DNA sequence variants by conformation-sensitive capillary electrophoresis. Genomics 2000;70:34–40.
18. Tian H, Brody LC, Fan S, Huang Z, Landers JP. Capillary and microchip electrophoresis for rapid detection of known mutations by combining allele-specific DNA amplification with heteroduplex analysis. Clin Chem 2001;47:173–85.
19. Kozlowski P, Krzyzosiak WJ. Combined SSCP/duplex analysis by capillary electrophoresis for more efficient mutation detection. Nucleic Acids Res 2001;29:E71.
20. den Dunnen JT, Antonorakis SE. Mutation nomenclature extensions and suggestions to describe complex mutations: a discussion. Hum Mutat 2000;15:7–12.
21. Geisel J, Walz T, Bodis M, Quast S, Herrmann W. Fluorescence-based SSCP analysis with automatic allele detection demonstrated for the factor V Leiden mutation [Technical Brief]. Clin Chem 1998;44:1986–7.
22. Andersen PS, Jespersgaard C, Vuust J, Christiansen M, Larsen LA. High-throughput single strand conformation polymorphism mutation detection by automated capillary array electrophoresis: validation of the method. Hum Mutat 2003;21:116–22.
23. Vallon-Christersson J, Cayanan C, Haraldsson K, Loman N, Bergthorsson JT, Brondum-Nielsen K, et al. Functional analysis of BRCA1 C-terminal missense mutations identified in breast and ovarian cancer families. Hum Mol Genet 2001;10:353–60.
24. Cartegni L, Chew SL, Krainer AR. Listening to silence and understanding nonsense: exonic mutations that affect splicing [Review]. Nat Rev Genet 2002;3:285–98.
25. Ganguly A. An update on conformation sensitive gel electrophoresis [Review]. Hum Mutat 2002;19:334–42.
26. Sevilla C, Moatti JP, Julian-Reyner C, Eisinger F, Stoppa-Lyonnet D, Bressac-de Paillerets B, et al. Testing for BRCA1 mutations: a cost effectiveness analysis. Eur J Hum Genet 2002;10:599–606.