Adaptation of Conformation-Sensitive Gel Electrophoresis to an ALFexpress™ DNA Sequencer to Screen *BRCA1* Mutations

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ABSTRACT

Conformation-sensitive gel electrophoresis (CSGE) has been introduced as the most reliable method for the screening of large and multi-exon genes because of its simplicity, sensitivity and specificity. Based on heteroduplex formation and with the use of mildly denaturing solvents, it allows detection of single-base mutations with accuracy. This is important in genes such as BRCA1 and BRCA2, in which alterations span the entire gene. We have adapted the CSGE assay to a fluorescent platform—a DNA sequencer one-color technology—that reduces the time involved and enhances resolving power for the complete scanning of the BRCA genes. Electrophoresis has high sensitivity and is performed in less than three hours, and the gel does not require staining with ethidium bromide. Eighteen single-base and six frameshift mutations in the BRCA1 gene were analyzed. We compared the manual and fluorescent CSGE methods, and all mutations were detected with accuracy.

INTRODUCTION

Conformation-sensitive gel electrophoresis (CSGE) is a heteroduplex-based technique that allows detection of single-base mismatches. This system differs from other heteroduplex-based methods in that mildly denaturing solvents are used (6).

Many genetic diseases show a high frequency of the so-called “private” mutations. Genetic testing of these diseases requires extensive screening procedures—often of the entire gene—to identify potential mutations. The rate-limiting step for identification of these unique mutations is exhaustive screening of all the coding exons and exon-intron boundaries for sequence variations, as in the case of the *BRCA1* and *BRCA2* genes responsible for the susceptibility to breast and ovarian cancer (10,12). To date, more than 600 distinct mutations, polymorphisms and variants have been identified in the *BRCA1* gene, of which approximately 400 are reported only once (Breast Cancer Information Core, BIC, http://www.nhgr.nih.gov/Intramural_research/Lab_transfer/Bic).

Screening *BRCA1* mutations is a long and complicated process that requires optimal conditions. The identification of subtle changes in DNA sequence, such as single-base substitutions, remains challenging in terms of speed, accuracy and cost effectiveness. Indeed, sequencing of the gene is the “gold standard” for identification of mutations but is not practical as a routine procedure in a clinical laboratory

Research Report

for scanning large, multi-exon genes. Alternative approaches include the use of scanning methods that allow the rapid and accurate analysis for the presence of alterations in either the coding sequences of the gene (including exons and exon-intron boundaries, which are most likely to have a mutation) or in the entire gene when the total length is rather short. When such analyses suggest a variation, sequencing can be carried out to confirm its presence and identify the mutation.

A comparison of a variety of screening methods for large and multi-exon genes suggests CSGE as an advantage for its speed and cost effectiveness (5). Its sensitivity for *BRCA* and other genes has been shown (1,3,4,8,9,11), and its simplicity makes the method even more attractive. Here, we report an adaptation of the CSGE mutation-scanning assay to a fluorescent platform (fluorescent-CSGE), the ALFlexpress™ DNA sequencer (Amersham Pharmacia Biotech, Uppsala, Sweden), that further improves the speed, resolving power and simplicity (although it does not use ethidium bromide) of the conventional CSGE.

Our results from the application of this method to the genetic analysis of *BRCA1* mutations confirm all the single-base substitutions and frameshift mutations previously characterized by direct sequencing.

MATERIALS AND METHODS

We tested 18 single-base and six frameshift mutations in the *BRCA1* coding region previously identified by direct sequencing. The alterations were located in exons 2, 7, 8, 10, 11, 13, 16 and 18. All were studied by conventional CSGE and fluorescent-CSGE to compare the sensitivity, specificity and resolving power of the methods (Table 1).

Generation of PCR Products

The exons and regions of interest were amplified (between 230 and 500 bp in length) using the Thermocycler® 9600 (PE Biosystems, Foster City, CA, USA) from genomic DNA. The sequences of primers for genomic DNA amplification were selected so that the

Table 1. *BRCA1* Mutations Analyzed by CSGE and Fluorescent-CSGE

Mutation	Exon ^a	CSGE		Fluorescent-CSGE	
		Bands	Resolving	Peaks	Resolving
185delAG	2	3	++	4	+++
IVS7-34C>T	17	2	++	2	++
IVS8-58delT	18	3	++	4	+++
IVS10-49delT	110	3	++	4	+++
Q356R	11.a	2	++	2	++
1623del5	11.b	3	++	4	+++
D693N	11.c	2	++	2	++
2201C>T	11.c	2	++	2	++
2274insA	11.c	3	++	4	+++
2430T>C	11.c	2	+	2	+
P871L	11.d	2	++	2	++
E1038G	11.e	2	+	2	++
S1040N	11.e	2	+	2	++
K1183R	11.f	3	++	3	+
N1236K	11.f	2	+	2	++
3875del4	11.f	3	++	4	+++
4077T>C	11.g	2	++	2	++
4427T>C	13	2	++	2	++
4808C>G	16	2	++	2	++
4952C>T/S1613G	16	2	+	2	++
S1613G	16	2	++	2	++
M1652I	16	2	+	2	++
A1708E	18	2	++	2	++
IVS18+66G>A	118	2	++	2	++

a|, intron
+ low; ++ medium; +++ high
Bold characters indicate those mutations that are detected with different resolving power—in terms of number of bands or peaks—depending on the method used.

PCR products encompassed the exon, plus 50 bases of the flanking introns, or in the case of exon 11, the region of interest plus 50 bases (Table 2). For the generation of fluorescent PCR products, each forward primer was labeled at the 5'-end with Cy5 dye (Amersham Pharmacia Biotech). As performed under standard conditions, the PCR mixture contained 100 ng genomic DNA; 2 µL 10× buffer (Roche Molecular Biochemicals, Mannheim, Germany); 2 µL 2 mM mixture of dATP, dTTP, dCTP, and dGTP; 1 µL 10 µM each primer; 0.2 mL *Taq* DNA polymerase (5 U/µL) (Roche Molecular Biochemicals) and

12.8 µL distilled water. Amplification conditions were as follows: denaturing at 94°C for 5 min followed by 30 cycles of denaturing at 94°C for 30 s, annealing at 55°C for 45 s, extension at 72°C for 1 min and a final extension at 72°C for 7 min. Heteroduplex formation between the normal and mutant alleles (if present) took place during the final PCR cycles.

Manual-CSGE

The method (6), gel and electrophoresis conditions (3,4) were described earlier.

Table 2. *BRCA1* Primers Used for Amplification of Exons and Fragments

Exon	Name	Sense (5'→3')	Name	Antisense (5'→3')	Product length (bp)
2	2F	GAAGTTGTCATTTTATAAACCTTT	2R	TGTCTTTTCTTCCCTAGTATGT	258
7	7F	CACAACAAAGAGCATACATAGGG	JB7R	GAAGAAGAAGAAGAAGAAAACAAATG	274
8	JB8F	GGAGGAAAAGCACAGAAC	JB8R	GCTGCCTACCACAAATAC	269
9	JB9F	CAACAAGTACATTTTTTTAAACC	JB9R	CTATACACCAAATCCCAAGTC	286
10	10F	TGGTCAGCTTTCTGTAATCG	10R	GTATCTACCCACTCTCTTCTTCAG	241
11.a	e11BF	CAACATAACAGATGGGCTGGAAG	e11BR	TGCAGGTTATGTAGTCGATGAAACC	296
11.b	e11DF	GAAAACCTATCGGAAGAAGGCAAG	e11DR	AGTAGTGAAGTGGTAAGACGAGG	253
11.c	e11GF	GCAACTGGAGCCAAGAAGAGTAAC	e11GR	GGACTCACGGTATTAGTCATGG	319
11.d	e11IF	GCACTCTAGGGAAGGCAAAAACAG	e11JR	TGGCTTATCTTTCTGACCAACCAC	449
11.e	e11LF	TCAATGTCACCTGAAAGAGAAATGG	e11LR	GTCCTACGAATGTTAATGAAGGTCC	301
11.f	e11NF	GTTTGTCTGAGACACCTGATGACC	e11OR	CACTACAAGGACTCTACGGAAAC	420
11.g	e11PF	CGTTGCTACCGAGTGTCTGTCT	e11PR	TCGGGCAAGGAGAAAGAAGTAG	314
13	13F	AATGGAAAGCTTCTCAAAGTA	13R	ATGTTGGAGCTAGGTCCTTAC	320
16	16F	AATTCTTAACAGAGACCAGAAC	16R	AAAACCTCTTCCAGAATGTTG	450
18	JB18F	GAGGCTCTTTAGCTTCTTAG	JB18R	AGGTGTTAAACGTTAGGTG	234

Fluorescent-CSGE

The polyacrylamide gel used for fluorescent-CSGE was the same as the regular gel (6) that consisted of 10% polyacrylamide [99:1 acrylamide: *1,4-Bis(acryloyl)piperazine* (Fluka Chemie, Buchs, Switzerland)], 10% (v/v) ethylene glycol (Sigma, St. Louis, MO, USA) and 15% formamide (Fluka Chemie) in 0.5× TTE buffer.

The specific modifications we introduced to this procedure include (i) an ALFexpress DNA sequencer that was used for DNA fragment separation and analysis and allowed us to use the same fluorochrome (Cy5) to label all the sense primers; (ii) electrophoretic conditions that were carried out as follows: pre-run at 45 W for 15 min at 30°C in 0.5× TTE buffer and run at 45 W for 3 h at 30°C; and (iii) gels were 0.5 mm thick and in a 40-well-comb. The sample loading mixture contained 1 µL PCR product, 0.5 µL dextran blue (50 mg/mL stock) and 0.5 µL freshly deionized formamide. Four lanes of each gel were loaded with the 50–500-

bp-size ladder (Amersham Pharmacia Biotech) labeled with Cy5. Lanes 5, 15, 25 and 35 were only loaded with size markers. Lanes 5 and 25 were loaded in the first batch and lanes 15 and 35 in the second. The lanes that were located between the size ladder lanes were loaded with samples corresponding to the same fragment in each batch. This allowed us to study one set of 72 samples of the same fragment or, that is, eight sets of nine samples of the same fragment, as long as the order of loading and the sizes of the fragments were adequately selected.

Data analysis was carried out using Fragment Manager™ software (Amersham Pharmacia Biotech). Heterozygotes were identified by the presence of more than one band for a specific PCR product. Ideally, in the case of heterozygotes, there should be four bands: two homoduplex molecules and two heteroduplex molecules. Homozygotes were identified by the shift in the mobility of the corresponding band. The number of distinctly migrating bands depended on the sequence of the mole-

cules and the sequence context of the nucleotide, which is different between the normal and the mutant allele.

RESULTS

The sensitivity of any mutational scanning method is determined by its ability to detect and distinguish between different single-base substitutions. The *BRCA1* gene has multiple polymorphisms in both the coding and the noncoding sequences. As a test to compare the effectiveness of the CSGE and fluorescent-CSGE methods, we selected 24 different alterations (Table 1), including polymorphisms, mutations and small insertions or deletions in the *BRCA1* coding and flanking intronic regions as previously identified by direct sequencing.

Figure 1 shows the analysis of 258 bp of two PCR products flanking exon 2 of the *BRCA1* gene amplified from the genomic DNA of two different individuals, with both CSGE (panel A) and fluorescent-CSGE (panel B) methods. The

Research Report

Figure 1. Detection of the 185delAG frameshift mutation in a 258-bp PCR product from exon 2 of *BRCA1*. (A) Mutational scan from a carrier (sample 1) and a control individual (sample 2) by manual CSGE. The carrier shows three bands, of which two (c and d) correspond to two heteroduplex and one (a) to two homoduplex forms. (B) Mutational scan by fluorescent-CSGE. The carrier (sample 1) shows four peaks, two homoduplexes (a and b) and two heteroduplexes (c and d). (C) DNA sequence electropherograms from carrier (sample 1) and control (sample 2). * indicates the position of the AG deletion.

Figure 2. Detection of a single-base substitution, Q356R (A₁₁₈₆→G₁₁₈₆), present in a 296-bp PCR product from exon 11 of *BRCA1*. (A) Mutational scan by manual CSGE from a carrier (sample 1) and a control individual (sample 2). The carrier shows two bands corresponding to homoduplex (a) and heteroduplex (b) forms. (B) Mutational scan by fluoroscent-CSGE. The control (sample 2) shows one peak (a). The carrier (sample 1) shows a peak (a) with a shoulder (b) caused by the polymorphism. (C) DNA sequence electropherogram for the carrier (sample 1) and control (sample 2). * indicates the position of the polymorphism showing two overlapping peaks.

upper fluorescent-CSGE scan (panel B, sample 1) displays four peaks (a, b, c and d) from the presence of a 185delAG mutation. In contrast, conventional CSGE (panel A, sample 1) shows only three bands (a, c and d) for the presence of this mutation, which indicates a lower resolving power. All of the polymorphisms analyzed by fluorescent-CSGE were detected showing a double peak or a split peak (Figure 2) except K1183R ($A_{3667} \rightarrow G_{3667}$), which shows three peaks (Figure 4). We could detect several polymorphisms in the same DNA fragment by a different shift of peaks. Figure 3 shows scans of three 450-bp PCR products flanking exon 16 of the *BRCA1* gene amplified from the genomic DNA of three different individuals. Sample 1 in fluorescent-CSGE (panel B) shows a peak (b) with a shoulder

(c) that corresponds to two polymorphisms together, $4952C \rightarrow T$ ($C_{4952} \rightarrow T_{4952}$) and $S1613G$ ($A_{4956} \rightarrow G_{4956}$), in the same individual. Another peak (a) with a shoulder (b) but with different mobility is shown in sample 2 of panel B, which corresponds to only the $A_{4956} \rightarrow G_{4956}$ polymorphism in a different individual. The variation of mobility in these individuals is more apparent in panel B (fluorescent-CSGE) than in panel A (manual CSGE).

We also analyzed another fragment containing a homozygote polymorphism. Here, the peak from the fragment with a homozygote polymorphism shows a mobility different from either the normal or heterozygote polymorphism fragments. Figure 4 shows a polymorphism common in our environment from three different individuals who

have the heterozygote K1183R ($A_{3667} \rightarrow G_{3667}$) and homozygote (G_{3667}) forms and the normal homozygote form (A_{3667}), sample 1, 2 and 3, respectively. The three forms of this polymorphism can be distinguished in both the manual CSGE, (panel A) and fluorescent-CSGE, (panel B) by the presence of three bands or peaks (heterozygote mutation) or one band or peak (homozygote mutation) shift. In this case, the resolving power using fluorescent-CSGE was lower than manual CSGE.

Therefore, all previously known polymorphisms and mutations in the 24 samples were detected by fluorescent-CSGE as unambiguous migration shifts. Thus, we report here that the fluorescent adaptation of CSGE was as sensitive and specific as manual CSGE for frameshift mutations and, even more in-

Figure 3. Detection of two polymorphisms together in a 450-bp PCR product from exon 16 of *BRCA1*, $4952C \rightarrow T$ ($C_{4952} \rightarrow T_{4952}$) and $S1613G$ ($A_{4956} \rightarrow G_{4956}$). Sample 1 has the two polymorphisms, sample 2 has only the $A_{4956} \rightarrow G_{4956}$ polymorphism and sample 3 is a control individual. (A) Mutational scan by manual CSGE; samples 1 and 2 show two bands with a different mobility pattern but are not clearly differentiated. (B) Mutational scan by fluorescent-CSGE; samples 1 and 2 show two peaks, each one presenting a clear difference in mobility pattern. One peak of sample 2 corresponds to the peak of the control (a). One peak of sample 1 corresponds to one peak of the sample 2 (b). Peak c of sample 1 does not correspond with anything. (C) DNA sequence electropherograms for the three samples; the position of the heterozygous single-base substitutions is indicated by *.

Research Report

teresting, for single-base substitutions. Moreover, the resolving power using fluorescent-CSGE has been enhanced.

DISCUSSION

In analyzing a number of different genes, we have gained wide experience with the use of the conventional CSGE method, particularly with more than 200 samples from women with a family history of breast and ovarian cancer (2). This allowed us to realize the high sensitivity and specificity of the method. Here, we report significant improvement from modifying the methodology to the fluorescent platform ALFexpress DNA sequencer that resulted in a high-throughput, faster and simpler method, with enhanced resolving power in screening mutations in large and multi-exon genes, such as *BRCA1* and *BRCA2*.

Recently, Ganguly et al. (7) adapted the CSGE technique to a fluorescent platform (in this case, the ABI 377 DNA sequencer) and reported its advantages in terms of speed, reproducibility and resolving power. These two variants of fluorescent-CSGE, although essentially similar, provide notable differences. From a technical point of view, the method proposed by Ganguly et al. offers the use of four-color technology that provides a high throughput that is clearly an advantage. However, in using one-color technology, a single well can be charged with different samples from a multiplex PCR, provided the PCR products vary enough in size to clearly differentiate the bands belonging to each product.

In addition, many laboratories have DNA sequencers with only one-color technology. An advantage of this strategy is that all the primers are labeled with the same dye (i.e., Cy5 in the case

of the ALFexpress DNA sequencer), which is useful for both PCR amplification and sequencing. This variant allows for changing the strategy of multiplex PCR at no additional cost.

Interestingly, the variant of fluorescent-CSGE reported here includes a series of modifications in the gel conditions, which reduces the running time to 3 h for fragments of approximately 500 bp. The optimized conditions previously described for screening *BRCA* mutations by manual CSGE were 400 V, 16 h (3,4) and by fluorescent-CSGE, 2000 V, 4.5 h (7). In addition, the throughput of the technique proposed here can be greatly increased by a second loading of the same gel 2 h after the first. Electrophoresis with the two loads is completed in 5 h. In the case of the present study of the *BRCA1* gene (which included the analysis of 22 coding exons, a total of 34 fragments), we tested up to six patients in one gel (204 products). It

Figure 4. Detection of the polymorphism K1183R ($A_{3667} \rightarrow G_{3667}$), in heterozygous and homozygous form, in a 420-bp PCR product from exon 11 of *BRCA1*. (A) Mutational scan by manual CSGE; the heterozygous form of the polymorphism (sample 1) shows three bands (a, b and c). The homozygous form (sample 2) shows one band with a pattern of mobility different from the control (sample 3). (B) Mutational scan by fluorescent-CSGE; the heterozygous form (sample 1) shows three peaks (a, b and c) similar to bands in manual CSGE. The homozygous form (sample 2) shows one peak (c) with a different mobility pattern from the control (3-a). (C) DNA sequence electropherograms for the three samples where the * indicates the position of the single-base substitution (if present).

was not necessary to change the conditions for the gel in the conventional CSGE because the quenching effect associated with the four-color fluorescent-CSGE variant was not found.

However, it is important to limit the amount of PCR product that is loaded on the gel because with an excess of DNA, there is a plateau effect on the top of the respective peak, which cannot be corrected by the fragment analysis software. This handicap can be avoided by reducing the number of cycles in the PCR to 30, rather than by making dilutions of the samples. In this way, 1 μ L product is an adequate amount to obtain a stylized peak.

As to the sensitivity of the fluorescent-CSGE, results indicate it is as sensitive as the manual CSGE. All mutations studied by CSGE and fluorescent-CSGE were also detected in blind samples, indicating a high specificity. The fluorescent-CSGE presents a greater resolving power as shown in Table 1. Figure 1 is a good example of the enhanced resolving power of the method we report. It shows four peaks corresponding to two homoduplex bands and two heteroduplex bands from a frameshift mutation, the ideal result that could be expected from any heterozygote change. Similar images were found for all the frameshifts where one or more bases were deleted or inserted.

Figure 3 is a good illustration of the high resolving power of this technique that is able to distinguish two single-base substitutions, four bases apart, by different pattern splitting. Figure 4 shows the ability to distinguish mutations as migration shifts in heterozygote or homozygote samples. In the case of the K1183R mutation, the electrophoresis distinguishes three bands in the heterozygote mutation: two correspond to the two homoduplex "hybrids", *aa'* and *bb'*, and the third band corresponds to the two heteroduplex hybrids, *ab'* plus *a'b*, where A(*aa'*) and B(*bb'*) are the two allele sequences. The homozygote mutation (lane 2) shows one band with a different mobility than the control individual (lane 3).

Finally, Figure 2 is an example of all single-base substitutions we detected that were previously identified by direct sequencing as peaks with a shoulder or

split peaks. Samples without mutations did not show migration shifts. This clearly demonstrates the specificity level of the fluorescent-CSGE method as we have reported in this paper.

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