Interpreting epidemiological research: blinded comparison of methods used to estimate the prevalence of inherited mutations in *BRCA1*

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Abstract
While sequence analysis is considered by many to be the most sensitive method of detecting unknown mutations in large genes such as *BRCA1*, most published estimates of the prevalence of mutations in this gene have been derived from studies that have used other methods of gene analysis. In order to determine the relative sensitivity of techniques that are widely used in research on *BRCA1*, a set of blinded samples containing 58 distinct mutations were analysed by four separate laboratories. Each used one of the following methods: single strand conformational polymorphism analysis (SSCP), conformation sensitive gel electrophoresis (CSGE), two dimensional gene scanning (TDGS), and denaturing high performance liquid chromatography (DHPLC). Only the laboratory using DHPLC correctly identified each of the mutations. The laboratory using TDGS correctly identified 91% of the mutations but produced three apparent false positive results. The laboratories using SSCP and CSGE detected abnormal migration for 72% and 76% of the mutations, respectively, but subsequently confirmed and reported only 65% and 60% of mutations, respectively. False negatives therefore resulted not only from failure of the techniques to distinguish wild type from mutant, but also from failure to confirm the mutation by sequence analysis as well as from human errors leading to misreporting of results. These findings characterise sources of error in commonly used methods of mutation detection that should be addressed by laboratories using these methods. Based upon sources of error identified in this comparison, it is likely that mutations in *BRCA1* and *BRCA2* are more prevalent than some studies have previously reported. The findings of this comparison provide a basis for interpreting studies of mutations in susceptibility genes across many inherited cancer syndromes.
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The first inherited cancer syndrome for which clinical molecular genetic testing became considered to be the "standard of care" was multiple endocrine neoplasia type 2 (MEN 2).[1][2] The germline mutations in the *RET* gene that are responsible for MEN 2 are limited in number; consequently, a variety of techniques that are of equivalent sensitivity and specificity could be used for detecting mutations.[3] In the last decade, additional autosomal dominant inherited cancer syndromes have become genetically characterised and clinical testing made available. One of the most common inherited cancer syndromes is the hereditary breast-ovarian cancer syndrome (HBOC), which is primarily attributable to two genes, *BRCA1* and *BRCA2*,[4] which together comprise approximately 15 700 nucleotides of open reading frame. To date, more than 1000 mutations of deduced or established clinical significance have been identified; these are distributed throughout the 48 coding exons and respective splice junctions of the two genes. Therefore, molecular diagnostic testing for HBOC as well as molecular epidemiological studies in most populations require analytical methods that are capable of identifying hundreds of distinct mutations distributed along the lengths of these relatively large genes (http://www.nhgri.nih.gov/Intramural_research/Lab_transfer/Bic).

Direct nucleotide sequence analysis is considered the gold standard for mutation detection for genes such as *BRCA1* and *BRCA2*. However, this is one of the most expensive methods for analysing genes, not only because of reagent costs but also because of the labour required to analyse the more than 15 000 data points that it generates. Thus, many laboratories that analyse *BRCA1* or *BRCA2*, particularly in the context of performing epidemiological studies requiring analysis of numerous samples, use gene "scanning" technologies to identify sequence variants in PCR amplicons in order to avoid labour and cost intensive sequencing of wild type exons.[5] Although clinical cancer geneticists around the world counsel

mutations derived from such studies, the sensitivity and specificity of these methods, and thus the accuracy of these data, have not been systematically evaluated. Since these estimates are used for patient management, the accuracy of data derived by these methods has substantial implications, and some[4] but not all such studies take into account the potential for error with such methods. Further, research laboratories engaged in large scale molecular epidemiological studies need to understand the potential sources of error in such methods in order to maximise their sensitivity and specificity.

In an effort to assist the clinical cancer genetics community to evaluate results from different methods used for diagnosing HBOC through mutation detection, we sought to compare the sensitivity, specificity, and cost efficiency of four common mutation scanning technologies for detecting 58 distinct mutations in the *BRCA1* gene. Two of the methods, single strand conformational polymorphism analysis (SSCP) and conformation sensitive gradient gel electrophoresis (CSGE), screen for mutations on the basis of conformational changes in PCR products induced by mutations when compared to the wild type. The other two methods, two dimensional gene scanning (TDGS) and denaturing high performance liquid chromatography (DHPLC), separate mutational variants on the basis of their melting temperatures (TDGS also includes a size separation). It is believed that the value of the information derived from this comparison of mutation scanning methods is not limited to detection of mutations in *BRCA1* but has implications for the analysis of other large genes as well.

Materials and methods

SAMPLES

Samples were selected and anonymised for blinded analysis by Myriad Genetic Laboratories. All samples had been analysed following the routine procedures used for diagnostic testing. DNA was first extracted by Proteinase K digestion from peripheral blood mononuclear cells isolated from each sample and then column purified (QIAGEN Inc, Chatsworth, CA, USA). Aliquots of DNA were amplified by polymerase chain reaction (PCR) using 35 M13 forward and reverse tagged primer pairs to cover coding exons 2-24 of *BRCA1* (although exon 4, like exon 1, is non-coding and no variants in either were included in the subsequent inter-laboratory comparison). The amplified products were each directly sequenced in forward and reverse directions using fluorescent dye labelled sequencing primers. Chromatographic tracings of each amplicon were analysed by a proprietary computer based review followed by visual inspection and confirmation. Genetic variants were detected by comparison with a consensus wild type sequence constructed for *BRCA1*. As part of routine analytic processing, all potential genetic variants had been independently confirmed by repeated PCR amplification of the indicated gene region(s) and sequence deter-

For the purposes of this study, "mutations" were defined as protein truncating and missense mutations located within exons 2-3 and 5-24 of *BRCA1* as well as intronic sequence alterations occurring no more than 20 bp proximal or 10 bp distal to the ends of these exons. Non-truncating genetic variants were excluded from consideration for the purposes of this study if they had been observed at an allele frequency of greater than 1% of a suitable control population with no evidence for significantly higher frequency in cases than controls, or if published data indicated absence of substantial clinical significance, or if they neither altered the amino acid sequence nor were predicted to affect exon splicing significantly.

The sample set consisted of 65 samples, including 50 that contained a total of 58 mutations of established or potential clinical significance and 15 additional samples in which no mutation had been identified through sequence analysis as above. The positive samples included 20 frameshift mutations (17 deletions, three insertions), 18 nonsense mutations, 15 missense mutations, and five mutations occurring in the non-coding regions adjacent to the beginning or end of the exon (table 1). All mutations and genetic variants were named according to a designated convention,[6] numbering the nucleotides from the first transcribed base of *BRCA1* GenBank entry U14680.

Ten µg of genomic DNA that remained after the completion of routine analysis by Myriad Genetic Laboratories were aliquotted to the participating laboratories per their stated requirements as follows: 4 µg each for SSCP and CSGE and 1 µg each for TDGS and DHPLC. A letter of agreement was provided to each participating laboratory that delineated the principles of the exercise, including the criteria by which sensitivity and specificity would be derived. Differences between laboratories limited to the names or cDNA locations of the mutations were not considered discrepancies for the purpose of this comparison. A Myriad Genetic Laboratories representative (TSF) provided the number and identity of the mutations to a designated representative of the Breast Cancer Information Core (BIC) (LCB) to whom all laboratories subsequently submitted their results. Only when each of the laboratories had completed and submitted final results to the BIC representative were the authors provided with each other's results.

SSCP

All coding regions and exon-intron boundaries of *BRCA1* were amplified from genomic DNA by PCR using either previously described sets of primers[7 8] or primer pairs designed in the Ostrander laboratory. PCR was carried out in 12.5 µl volumes with 25 ng genomic DNA as template, $1 \times$ PCR buffer, 1.5 mmol/l magnesium, 0.048 mmol/l each dATP, dTTP, and dGTP, 0.0048 mmol/l dCTP, 0.2 U *Taq*, (Bioline, USA), and 0.004 mCi [α-P32]) dCTP (Amersham, USA). Initial denaturation was

Table 1   Mutations subject to blinded analysis by SSCP, CSGE, TDGS, and DHPLC

Mutation name	Exon	Base change	Mutation type	Mutation effect
187delAG	2	Del AG	Frameshift	Premature stop
M1I (122G>T)	2	122 G>T	Missense	Missense
C64Y	5	310 G>A	Missense	Missense
C61G	5	300 T>G	Missense	Missense
IVS5-11T>G	6	4795-11T>G	Splice	Splice
E143X	7	546 G>T	Nonsense	Nonsense
525insA	7	Ins A	Frameshift	Premature stop
Y179C*	8	655 A>G	Missense	Indeterminate
639delC	8	Del C	Frameshift	Premature stop
IVS8+1G>T	8	666+1 G>T	Splice	Splice
1629delC	11	Del C	Frameshift	Premature stop
2576delC	11	Del C	Frameshift	Premature stop
K679X	11	2154 A>T	Nonsense	Nonsense
L246V*	11	855 T>G	Missense	Indeterminate
F486L*	11	1575 T>C	Missense	Indeterminate
N550H*	11	1767 A>C	Missense	Indeterminate
E1222X	11	3783 G>T	Nonsense	Nonsense
E1134X	11	3519 G>T	Nonsense	Nonsense
Q1111X	11	3450 C>T	Nonsense	Nonsense
Q957X	11	2988 C>T	Nonsense	Nonsense
3600del11	11	Del GAAGATACTAG	Frameshift	Premature stop
1294del40	11	Del 40	Frameshift	Premature stop
V772A*	11	2434 T>C	Missense	Indeterminate
E1250X	11	3867 G>T	Nonsense	Nonsense
Q780X	11	2457 C>T	Nonsense	Nonsense
L668F*	11	2121 C>T	Missense	Indeterminate
2322delC	11	Del C	Frameshift	Premature stop
3347delAG	11	Del AG	Frameshift	Premature stop
E908X	11	2841 G>T	Nonsense	Nonsense
2072del4	11	Del GAAA	Frameshift	Premature stop
2080delA	11	Del A	Frameshift	Premature stop
W321X	11	1081 G>A	Nonsense	Nonsense
2594delC	11	Del C	Frameshift	Premature stop
3171ins5	11	Ins TGAGA	Frameshift	Premature stop
3829delT	11	Del T	Frameshift	Premature stop
3875del4	11	Del GCTC	Frameshift	Premature stop
4154delA	11	Del A	Frameshift	Premature stop
Q563X	11	1806 C>T	Nonsense	Nonsense
Q1240X	11	3837 C>T	Nonsense	Nonsense
2190delA	11	Del A	Frameshift	Premature stop
Q1395X	12	4302 C>T	Nonsense	Nonsense
H1402Y*	13	4323 C>T	Missense	Indeterminate
Y1463X	14	4508 C>A	Nonsense	Nonsense
4510del3insTT	14	Del CTA Ins TT	Frameshift	Premature stop
W1508X	15	4643 G>A	Nonsense	Nonsense
P1637L*	16	5029 C>T	Missense	Indeterminate
IVS16+1G>A	16	5105+1G>A	Splice	Splice
IVS17+1G>T	17	5193+1 G>T	Splice	Splice
A1708E	18	5242 C>A	Missense	Missense
Y1703X	18	5228 T>G	Nonsense	Nonsense
IVS17-1G>A	18	5194-1 G>A	Splice	Splice
5385insC ("5382insC")	20	Ins C	Frameshift	Premature stop
E1754X	20	5379 G>T	Nonsense	Nonsense
M1775R	21	5443 T>G	Missense	Missense
C1787S*	22	5478 T>A	Missense	Indeterminate
G1788D*	22	5482 G>A	Missense	Missense
5454delC	22	Del C	Frameshift	Premature stop
R1835X	24	5622 C>T	Nonsense	Nonsense

*The following groups of mutations were concurrently present in their respective samples: C1787S and G1788D; 1294del40 and V772A; 5385insC and H1402Y; Y1703X and L668F; 2576delC and P1637L; K679X and L246V; Y179C, F486L, and N550H.

cycles of 30 seconds at 94°C, 15 seconds at an appropriate annealing temperature, 15 seconds at 72°C, followed by final elongation at 74°C for three minutes. Samples were then diluted 1:3 in formamide buffer (98% formamide, 10 mmol/l EDTA, pH 8, 0.05% bromophenol blue, and 0.05% xylene cyanol), denatured at 99°C for five minutes, immediately placed on ice, and loaded on two types of gels, multiplex 0.5 MDE and non-multiplex 3% glycerol. Selected amplicons were then pooled for electrophoresis; this allowed simultaneous analysis of several fragments chosen according to band size and migration patterns. Electrophoresis was performed at room temperature for 16 to 20 hours at 6 W and eight hours at 8 W for MDE and glycerol gels, respectively. Results were visualised by autoradiography. Amplification and electrophoresis were re-

patterns. Variant bands were subsequently cut from gels, resuspended in distilled water, resubjected to PCR, and then sequenced with Big Dye Terminator Cycle Sequencing kits (PE Applied Biosystems) in both forward and reverse strand directions.

Following the routine practice of the laboratory, each abnormally migrating fragment was subject to sequence analysis regardless of whether it was also the location of a common *BRCA1* polymorphism, since it has been shown that the abnormal SSCP migration associated with common polymorphisms may mask a coexistent deleterious mutation.[9]

CSGE

The entire coding region of *BRCA1* including at least 15-50 bp of each flanking intron was subdivided into 33 segments. To facilitate PCR multiplexing and direct sequencing of selected fragments afterwards, all forward primers were tagged with M13-Forward tails and labelled with fluorescent FAM, HEX, or TET. Reverse primers contained M13-Reverse tails. Oligonucleotides were purchased from Eurogentec, Belgium; their sequences are available from the Devilee lab website (http://www.medfac. leidenuniv.nl/lab-devilee/Lab/csgeolig.htm).

The 33 fragments were amplified in one mono and 16 duplex PCRs as detailed on the website provided above. A 14 µl reaction mixture prepared in each well of a 96 well microtitre plate contained 10 pmol primers, 1 × PCR buffer (50 mmol/l KCl, 10 mmol/l TRIS-HCl, pH 8.4, 2.5 mmol/l MgCl$_2$, 0.2 mg/ml BSA, 0.2 mmol/l dNTPs), and 0.1 U Goldstar *Taq* polymerase (EuroGentech, Seraing, Belgium). Subsequently, 1 µl of each DNA sample (50 ng/µl) was added to the reaction mixtures. PCR was performed for 40 cycles consisting of 30 seconds at 94°C, 30 seconds at 58°C, and 30 seconds at 72°C.

After PCR, reaction mixtures corresponding to a given DNA sample were pooled into a 96 well microtitre plate in a HEX:FAM:TET ratio of 3:2:2 for a final volume of 24 µl, in a total of six pools per DNA sample (see above website for details). Seven µl of this mixture were aliquotted into a fresh plate and heat/air dried by exposing to 45°C for one hour. The mixture was dissolved in 2.5 µl of Pink Loading Dye (Amersham Pharmacia, Benelux, Roosendaal, The Netherlands), to which 0.25 µl GeneScan-500 TAMRA size standard and 0.25 µl loading buffer were added (Applied Biosystems). Using an eight channel loading device (Hamilton, Bonaduz, Switzerland), 1.5 µl of this mixture was loaded onto an f-CSGE gel, which had been pre-run for 15 minutes. The samples were subjected to electrophoresis through these gels for 4.5 hours at 1680 V at 30°C. Gels were analysed with GeneScan® and Genotyper® software (Applied Biosystems). Each abnormally migrating fragment was reamplified from the DNA sample using the same primers as above and sequenced in the forward direction using Big Dye Terminator Cycle Sequencing kits (PE Applied Biosystems).

TDGS

All *BRCA1* coding exons were amplified from genomic DNA in a 7-plex long distance PCR. Individual exons or parts of exons were amplified in four multiplex groups of nine or 10 fragments each, using the long distance 7-plex PCR products as template, so that the entire *BRCA1* coding region was resolved in a total of 37 fragments. Primers for the multiplex short PCR were designed as described.[10][11] Products of the four multiplex groups were combined, mixed with sample buffer, and loaded directly into the slot of a 2D gel. Electrophoresis was performed in an automated 2D DNA electrophoresis system[12] and gels were stained with ethidium bromide. Spot patterns were interpreted by eye for the appearance of four spots rather than one, indicating the presence of a heterozygous mutation or polymorphism. The complete protocol for *BRCA1*-TDGS has been described previously.[11] Each sample was analysed only once, under the same conditions, and fragments that were absent or faint were repeated by one dimensional DGGE (an average of five fragments per *BRCA1* gene sample). Fragments that showed a four spot pattern that could be recognised as a previously detected polymorphism on the basis of their characteristic configurations were assigned as such. New variants were subjected to sequence analysis. Sequence analysis was either carried out on a Beckman CEQ2000 sequencer (75% of fragments) or contracted out to DavisSequencing (Davis, CA, USA) (25% of fragments). All 2D patterns are published on the web (http://www.tdgs.saci.org/myriad.html).

DHPLC

For purposes of PCR, *BRCA1* was divided into 35 amplicons comprising the coding sequence and adjacent non-coding sequence in the regions of the splice junctions. Primers were designed to minimise overlap between fragments, to improve the robustness of PCR, or to increase the length of fragment screened. The primers used had originally been described for SSCP[13] with the exception of the primers for exon 5.[8] PCR was performed in a 50 µl volume containing 15 mmol/l Tris-HCl, pH 8.0, 50 mmol/l KCl, 1.5-4.5 mmol/l $MgCl_2$, 10 mmol/l of dNTPs, 0.25 µmol/l of each primer, and 10 ng of genomic DNA. For all PCR reactions, Ampli*Taq* Gold (Perkin Elmer, Foster City, USA) was used. The PCR cycling conditions comprised an initial denaturation step at 95°C for seven minutes to activate Ampli*Taq* Gold. Subsequent denaturing steps were 94°C for 45 seconds and extension steps of 72°C for 30 seconds. In some instances, annealing temperatures were decreased from 63°C by 0.5°C decrements to 56°C in 14 cycles, followed by 21 cycles at 56°C for 20 seconds. In one case, namely exon 23, the annealing temperature was decreased from 67°C to 60°C, while in the case of exon 11EF, it was decreased from 65°C to 58°C. In all other cases, 35 cycles were performed at constant annealing temperatures.

Denaturing high performance liquid chromatography was carried out on an automated HPLC instrument (Transgenomics Inc, San Jose, CA, USA). The DNA separation column was packed with proprietary 2 µ non-porous alkylated poly(styrene-divinylbenzene) particles.[14] The mobile phase was 0.1 mol/l triethylammonium acetate buffer, pH 7.0 (TEAA, PE Biosystems, Foster City, CA, USA). Crude PCR products were subjected to an additional three minute, 95°C denaturing step followed by gradual reannealing from 95-65°C over a period of 30 minutes before analysis. Homo- and heteroduplex species were eluted with a linear acetonitrile (Merck, Vienna, Austria) gradient at a flow rate of 0.9 ml/minute. The start and end points of the gradient were adjusted according to the size of the PCR products using an algorithm provided by the WAVE Maker™ system control software (Transgenomics Inc, San Jose, CA, USA). Generally, analysis took eight minutes, including column regeneration and re-equilibration to the starting conditions. The temperature required for successful resolution of heteroduplex molecules was determined by use of the DHPLC melting algorithm available at http://insertion.stanford.edu/melt.html,[15] respectively, the WAVE Maker™ software. Appropriate temperature(s) of analysis were determined for each amplicon, with 19 of the 35 amplicons requiring analysis at two temperatures and the rest at one. Known sequence variants, on average four per amplicon, are analysed along with the new samples to establish the proper performance of the DHPLC instrument. The appearance of additional peaks or shoulders was interpreted as indicative of the presence of a mismatch, which was subsequently analysed by sequencing. Nine amplicons known to contain *BRCA1* polymorphisms with a heterozygosity ≥5% were sequenced routinely when observed to be heterozygous.[8]

COST CALCULATION

The costs of mutation analysis were calculated in two ways. The first only took into account the cost of consumable supplies on a per sample basis. The second calculation derived a "universal cost equivalent" that attempts to analyse each method in terms of labour, quantities of supplies (for example, numbers of ABI gels, numbers of oligonucleotide primers, number of PCR reactions) and run times necessary to perform an analysis.

Results

For the set of samples known to contain *BRCA1* mutations or not (table 1), the reported overall sensitivity of the methods, as summarised in table 2, required not only the initial detection of an abnormality in an amplicon, but also the ability to confirm the mutation by sequence analysis and to report the result correctly to a central source (LCB) who compiled the results. Samples for which PCR amplification could not be attained or for which there was insufficient DNA for sequence confirmation were not counted as "negative" results, but were omitted from the total. Only DHPLC was able to correctly identify each of the 58 mutations in the sample set. Eleven

Table 2   Comparison of methods for detecting mutations in BRCA1*

Mutation type (number in set) *	Abnormal migration (%)	Confirmation of mutation in abnormally migrating fragment (%)	Total mutations reported correctly (%)
(A) SSCP			
Frameshift (20)	19/20 (95)	17/18 (94)	16/19 (84)
Base substitutions			
Nonsense (18)	7/18 (39)	7/7 (100)	7/18 (39)
Missense (15)	12/15 (80)	7/7 (100)	7/10 (70)
Splice (5)	4/5 (80)	3/3 (100)	3/4 (75)
Total	42/58 (72)	34/35 (97)	33/51 (65)
(B) CSGE			
Frameshift (20)	14/15 (93)	11/14 (79)	10/15 (67)
Base substitutions			
Nonsense (18)	8/12 (67)	8/8 (100)	6/12 (50)
Missense (15)	10/15 (67)	9/10 (90)	9/15 (60)
Splice (5)	2/3 (67)	2/2 (100)	2/3 (67)
Total	34/45 (76)	30/34 (88)	27/45 (60)
(C) TDGS			
Frameshift (20)	18/20 (90)	18/18 (100)	18/20 (90)
Base substitutions			
Nonsense (18)	17/18 (94)	16/16 (100)	16/17 (94)
Missense (15)	14/15 (93)	14/14 (100)	14/15 (93)
Splice (5)	4/5 (80)	3/3 (100)	3/4 (75)
Total	53/58 (91)	51/51 (100)	51/56 (91)
(D) DHPLC			
Frameshift (20)	20/20 (100)	20/20 (100)	20/20 (100)
Base substitutions			
Nonsense (18)	18/18 (100)	18/18 (100)	18/18 (100)
Missense (15)	15/15 (100)	15/15 (100)	15/15 (100)
Splice (5)	5/5 (100)	5/5 (100)	5/5 (100)
Total	58/58 (100)	58/58 (100)	58/58 (100)

*Discrepant values between the number of samples in the set and the number of samples analysed (denominator) reflect samples for which either PCR amplification failed or for which there was insufficient DNA for sequence analysis following initial screening.

laboratories, of which two (both single nucleotide substitutions resulting in premature truncation) were each missed by three of the four laboratories. The results of the individual laboratories are presented below.

SSCP
The laboratory using SSCP correctly identified 33 of 51 mutations (65%) (table 2A), with seven additional mutations occurring in samples that could not be analysed because of insufficient DNA for sequence analysis following SSCP, as discussed below. A false positive result that was reported in one of the 15 negative samples resulted not from a technical error, but instead from a laboratory sample switch that also accounted for one of the false negative results.

After the initial SSCP scan, 58 aberrant bands were detected on MDE gels and an additional five bands were observed on glycerol gels. Initially, two of five variants seen on glycerol gels were not detected on MDE gels owing to the presence of overlapping multiplex bands representing other exons. Reamplification of all possible variants from the initial screen confirmed the presence of 42 abnormal bands out of the 58 mutations (72%) distributed in different exons of *BRCA1*. Aberrant electrophoresis of bands was identified for 19 of 20 (95%) frameshift mutations, including 17 of 17 deletions and two of three insertions. The mutation 5385insC ("5382insC") was missed, although it has been previously detected by SSCP by this laboratory using the same techniques.[16–18] This suggests that the efficiency of SSCP in detecting very subtle changes is

of 38 nucleotide substitutions (61%), including five localised to introns (table 2A). SSCP failed to detect seven of the nine G to T substitutions and four of the 10 C to T substitutions, but abnormal migration occurred with five of six G to A substitutions. Several of the single nucleotide substitutions that did not alter electrophoresis mobility occurred near either end of a PCR amplicon. For example, the missed G to T substitutions that resulted in M1I and E143X each occurred near the ends of exon 2 and exon 7, respectively.

Sequence analysis was performed for all samples for which abnormal migration was identified. In seven instances, the first obtained sequence was not diagnostic and there was insufficient DNA to repeat the procedure; these seven variants were excluded from further calculations of sensitivity. Sequence analysis identified 34 of the remaining 35 mutations for which abnormal migration had been observed, but failed to identify the frameshift mutation 2576delC. Finally, as mentioned above, mislabelled samples resulted in incorrect reporting of two samples, resulting in one false positive and one false negative interpretation in the final report of results.

CSGE
The laboratory using CSGE correctly identified 27 of 45 mutations (60%), with 13 mutations that could not be analysed owing to failure to amplify by PCR as discussed below (table 2B). No mutations were identified in the 15 samples documented not to harbour a sequence alteration.

Abnormal electrophoretic migration by CSGE was present in 34 of 45 (76%) samples for which PCR amplification was successfully performed. Nucleotide substitutions accounted for 10 of the 11 mutations that were missed at this stage of analysis (table 2B). Retrospective evaluation showed subtle differences relative to wild type fragments in three of these 11 false negatives, and two additional peak shifts were sufficiently clear that they represented erroneous interpretation by the observer. The remaining six (all missense changes: G>T, G>T, C>T, T>C, C>A, A>T) did not show migration patterns that were distinguishable from the wild type.

Four additional mutations were missed because of failure of sequence analysis to confirm a mutation following observation of abnormal gel mobility. One of these was a base substitution, a T to G at cDNA nt 855, resulting in the substitution of valine for leucine at amino acid position 246. This variant produced only a very subtle change in the sequence trace at the heterozygote position and was erroneously called negative. The other three were small frameshifting deletions (2072del4, 2080delA, and 2594delC) that are ordinarily considered to be easily detectable by sequencing. Sequence data were analysed using the Staden software, which subtracts the sample sequence trace from a wild type control trace to highlight sequence differences, exclud-

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