

METHODS

Comparison of DNA- and RNA-Based Methods for Detection of Truncating *BRCA1* Mutations

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A number of methods are used for mutational analysis of *BRCA1*, a large multi-exon gene. A comparison was made of five methods to detect mutations generating premature stop codons that are predicted to result in synthesis of a truncated protein in *BRCA1*. These included four DNA-based methods: two-dimensional gene scanning (TDGS), denaturing high performance liquid chromatography (DHPLC), enzymatic mutation detection (EMD), and single strand conformation polymorphism analysis (SSCP) and an RNA/DNA-based protein truncation test (PTT) with and without complementary 5' sequencing. DNA and RNA samples isolated from 21 coded lymphoblastoid cell line samples were tested. These specimens had previously been analyzed by direct automated DNA sequencing, considered to be the optimum method for mutation detection. The set of 21 cell lines included 14 samples with 13 unique frameshift or nonsense mutations, three samples with two unique splice site mutations, and four samples without deleterious mutations. The present study focused on the detection of protein-truncating mutations, those that have been reported most often to be disease-causing alterations that segregate with cancer in families. PTT with complementary 5' sequencing correctly identified all 15 deleterious mutations. Not surprisingly, the DNA-based techniques did not detect a deletion of exon 22. EMD and DHPLC identified all of the mutations with the exception of the exon 22 deletion. Two mutations were initially missed by TDGS, but could be detected after slight changes in the test design, and five truncating mutations were missed by SSCP. It will continue to be important to use complementary methods for mutational analysis. *Hum Mutat* 20:65–73, 2002. © 2002 Wiley-Liss, Inc.

KEY WORDS: *BRCA1*; molecular testing; two-dimensional gene scanning; TDGS; DHPLC; enzymatic mutation detection; EMD; single strand conformation polymorphism analysis; SSCP; protein truncation test; PTT

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DATABASES:

BRCA1 – OMIM: 113705; GenBank U14680

BRCA2 – OMIM: 600185; GenBank U43746

www.nhgri.nih.gov/Intramural_research/Lab_transfer/Bic/Member/index.html (Breast Cancer Information Core (BIC) database)

INTRODUCTION

The accepted deleterious mutations in *BRCA1* (MIM# 113705) and *BRCA2* (MIM# 600185) are predominantly frameshift or nonsense mutations that truncate the *BRCA1* and *BRCA2* gene products (protein truncating mutations) and result in loss of function of the proteins. Single base substitutions that result in amino acid changes in the *BRCA1* and *BRCA2* genes have also been reported to segregate in families, although the frequency of these mutations that are clinically significant is lower than the protein-truncating mutations. The complete analysis of both *BRCA1* and *BRCA2* is difficult because the types of mutations are variable and their locations are distributed throughout the large coding sequences. Currently, we are far from identifying all of the different mutations in the gene, clarifying their effects on protein function, and determining how each contributes to the disease phenotype.

Reported *BRCA1* mutation frequencies are influenced by multiple technical factors including: detection methodology, accuracy of individual laboratories using the same detection strategy, and whether the full gene or specific DNA fragments are examined. The analysis of large multi-exon genes for the presence of sequence variations is a very complex and laborious task. PCR amplification of all coding regions and adjacent non-coding sequences must be followed by a specific screening technology. The optimum method has been considered to be direct automated DNA sequencing. However, the large costs in both equipment and reagents make this method untenable for the average investigator. Moreover, the screening of a large number of samples exacerbates the problems. Due to these difficulties, several other methods have been adopted.

The purpose of the present study was to determine the sensitivity and specificity of several DNA-based methods and an RNA/DNA-based method to detect truncating mutations in *BRCA1* (relative to DNA sequencing). While the majority of reported alterations of *BRCA1* and *BRCA2* that have a functional impact are protein-truncating mutations, a significant fraction are missense changes. In this study, we have focused on the detection of protein-truncating mutations that have been reported to be disease-causing alterations that segregate with cancer in families. The study involved testing a set of DNA and RNA samples isolated from 21 coded lymphoblastoid cell line samples in seven laboratories using five

different techniques. The *BRCA1* mutation status of the cell lines had been determined previously by direct DNA sequencing analysis, considered to be the optimum method for mutation detection.

MATERIALS AND METHODS**Preparation of Standards for Mutation Analysis**

The cell lines were established from peripheral blood lymphocytes from individuals in high-risk breast/ovarian cancer families using standard Epstein-Barr virus transformation protocols, which include cell separation by gradient centrifugation and use of the T-lymphocyte cytotoxin cyclosporin A [Beck et al., 2001]. These biospecimens are particularly useful since they can be used for the preparation of both DNA and RNA required for the various techniques. Once the mutations were verified by direct DNA sequencing analysis, the cell lines were transferred to the Coriell Institute where they were assigned a code number and expanded to produce sufficient material for distribution. High molecular weight DNA was isolated using a modification of the procedure of Miller et al. [1998]. The identity of the DNA sample was verified by gender analysis and the use of six highly polymorphic tetranucleotide repeat microsatellite markers with sufficiently high heterozygosities that the probability of identical profiles for unrelated individuals using these markers is 1 in 33 million. Coded DNA and frozen cell pellets were distributed to the seven laboratories for mutational analysis.

BRCA1 Genotyping Methods

Each of these methods is designed to detect the presence of a variation in sequence. DNA sequencing is then performed to identify the specific change.

Enzymatic Mutation Detection (EMD)

The EMD method uses a resolvase, endoVII, cloned from bacteriophage T4 [Del Tito et al., 1998]. It is an enzyme known to have high specificity for insertions, deletions, and base-substitution mismatches. PCR is used to amplify the normal and mutant alleles of the target sequence. The forward PCR primers are labeled with FAM (6-carboxyfluorescein amidite), a blue fluorescent dye, and the reverse primers with TET (4, 7, 2', 7' – tetrachloro-6-carboxyfluorescein amidite, a green fluorescent dye. Upon denaturing and renaturing of the normal and mutant allele in a mixture, mismatch heteroduplexes will be formed approximately 50% of the time. For each base change, two mismatches are formed. The endoVII enzyme scans along the double-stranded DNA until it detects a structural distortion, either a bubble caused by single base pair mismatches or a heteroduplex loop formed by hybridizing a wild-type allele with a mutant allele containing an insertion or deletion [Del Tito et al., 1998]. The enzyme cleaves within 6 bp on the 3' side of the mutation forming two shorter fragments, one blue and one green. The DNA product is analyzed on an

automated DNA sequencer and the mobility of each fragment is evaluated.

Genomic DNA samples potentially heterozygous for mutations in specific exons of *BRCA1* were amplified by PCR for each individual exon of the *BRCA1* gene including intron/exon boundaries. The amplified products ($n = 30$) ranged in size from 209 to 570 bp.

Enzymatic digestion was performed according to manufacturer's instruction (Amersham Pharmacia Biotech, Piscataway, NJ) with minor modification. The reactions were incubated at 37°C (30 min) and stopped with formamide containing 0.5ul GeneScan ROX molecular weight marker (500 bp; ABI, Foster City, CA). Enzyme reaction samples were placed immediately at -20°C; optimal conditions call for the samples to be resolved by capillary electrophoresis as soon as possible, but this can be delayed if maintained without thawing at -20°C. The DNA product were resolved on an automated DNA sequencer, ABI Model 3100 using a 36 cm capillary array. The mobility of each fragment was analyzed with Prisma GeneScan 3.5 software (ABI). Positive and wild-type controls were run with test samples for each exon.

Two-Dimensional Gene Scanning (TDGS)

The use of two-dimensional gene scanning for detection of germline mutations in cancer susceptibility genes, including *BRCA1*, p53, and *RBI*, has been described in a series of published reports [Dhanda et al., 1998a,b; Li and Vijg, 1996; Rines et al., 1998; Van Orsouw and Vijg, 1999; Van Orsouw et al., 1996, 1998, 1999]. A customized computer program [Van Orsouw et al., 1998] was used to design optimal primer combinations for PCR amplification, denaturing gradient gel electrophoresis and DNA-fragment distribution in the 2-D gel. The resulting test permits the detection of mutations and polymorphisms in *BRCA1* coding regions as well as splice site mutations. The entire *BRCA1* coding region and part of the introns was amplified in a 2-step PCR process requiring only six individual multiplex PCR reactions (six tubes). Initially, one multiplex PCR-amplification was performed to generate seven long PCR fragments that contain all *BRCA1* exons. Using these products as template, individual exons were amplified in four multiplex groups to generate 37 fragments of between 100 and 500 bp, the optimal size for detecting mutations. PCR was completed by one round of denaturation/renaturation to achieve heteroduplexing of heterozygous mutations or polymorphisms. The products were then combined, directly loaded onto an automated 2-D gel electrophoresis instrument (Accelerated Genomics Inc., San Antonio, TX), and separated by fragment size and migration in a denaturing gradient gel (melting). Different mutations or polymorphisms in the same fragment can be recognized on the basis of spot configuration, i.e., variously spaced homoduplex and heteroduplex fragments. This obviates the need for sequence confirmation of recurrent mutations or polymorphisms [Van Orsouw et al., 1999]. When results of the 2-D gel analyses showed aberrant patterns or missing DNA-spots, the specific fragment was re-examined in a one-dimensional gel and the aberrant fragment was sequenced to identify the DNA alteration.

Protein Truncation Test (PTT)

Mutational analysis of the complete coding region of *BRCA1* was performed using PTT in two participating laboratories. Both laboratories used both RNA and DNA as a PCR template, achieved protein synthesis from the PCR template using a coupled transcription-translation in vitro system and radio-labeled protein products, allowing shorter protein

products to be detected via autoradiography [Den Dunnen and Van Ommen, 1999; Özçelik et al., 1996; Southey et al., 1999; Yazici et al., 2000]. One laboratory performed the *BRCA1* analysis with three and four overlapping PCR fragments using DNA and RNA as a template, respectively [Özçelik et al., 1996]. Since mutations in the 5' region of the gene result in protein products that are too small to be readily detectable by PTT alone, one laboratory used additional complementary sequencing analysis of exons 2, 3, 5, and 6 (PTT+), which was performed using the ABI 377 automated sequencing system according to the manufacturer's protocols. The second laboratory performed the *BRCA1* PTT analysis using five overlapping PCR fragments only, and results of the laboratories were compared. All putative protein-truncating mutations were confirmed using automated or manual sequencing approaches.

Single Strand Conformation Polymorphism Analysis (SSCP)

Two laboratories used modified SSCP approaches for the screening of 22 coding exons of *BRCA1* in addition to intronic splice-donor and -acceptor regions. Briefly, the denatured amplified products were electrophoresed on non-denaturing gels prepared with $0.5 \times$ MDE (mutation detection enhancement) (AT Biochem) in $0.6 \times$ TBE buffer at 6W for 12 to 16 hr at room temperature, depending on amplicon size. The gels were dried and exposed to x-ray film. When a variant was identified as an aberrant fragment, it was sequenced in both directions to identify the actual mutation. In the first lab, the methods and 37 amplicons with primers were previously described [Takahashi et al., 1995, 1996]. In the second lab, for complete mutation screening of the coding regions and intron-exon boundaries of *BRCA1*, 44 amplicons for *BRCA1* were examined by SSCP. Primers were designed so that amplicons were no more than 300 bp and there was overlap of approximately 50 bp for each set of amplicons.

DHPLC

Forty-two PCR products were generated, which covered the entire gene. The average length of each product was between 300 and 500 bp (many of these fragments are essentially as described by Gross et al. [2000]). After PCR amplification, samples were loaded directly into a Transgenomic Wave DHPLC machine (Omaha, NE), which then loaded samples onto the column automatically; no PCR cleanup is necessary for DHPLC analysis, only for subsequent sequencing.

Statistical Analysis

The individual laboratories sent the results of their *BRCA1* mutational analyses to one of us (A.S.W.) for computation of method-specific sensitivity and comparison of sensitivities across the six methods (PTT, EMD, TDGS, DHPLC, SSCP, and DNA sequencing). Sensitivity was computed as the number of unique mutations detected/total number of unique mutations ($n = 15$).

RESULTS AND DISCUSSION

BRCA1 and *BRCA2* Mutational Analysis

The aim of the study was to evaluate methods of detecting *BRCA1* mutations that are likely to be disease-causing. A series of previously established cell lines with *BRCA1* mutations were chosen by two of us (S.B. and A.W.) to comprise the validation set for this

TABLE 1. Types of Mutations Potentially Detected by Various Methods

Techniques	Protein-truncating mutations	Missense mutations/polymorphisms	Large genomic deletions/insertions
SSCP/SSCA	Yes	Yes	No
2-D gel scanning	Yes	Yes	No
EMD	Yes	Yes	No
PTT	Yes	No	Yes
DHPLC	Yes	Yes	No
DNA Sequencing	Yes	Yes	No

*Small deletions/insertions within an exon.

**Deletions/insertions affecting a single or few exons.

study. Since most of the reported alterations of *BRCA1* that have a functional impact are protein-truncating mutations, we compared the sensitivities of the techniques to detect this type of mutation. The laboratories were blinded to the *BRCA1* mutation status of the samples until after the statistical analysis.

In Table 1, the types of mutations that can be detected by the various techniques used in this study are shown. All five methods can detect various protein-truncating mutations. All methods except for PTT can detect missense mutations and polymorphisms, whereas PTT is the only method used in this study that can identify large insertions or deletions that cause protein truncation.

Detection of Protein-Truncating Mutations

The set of 21 cell lines included 10 samples with frameshift mutations, four with nonsense mutations

(three unique), and three with splice-site mutations (two unique), as well as four specimens without truncating mutations (Table 2). Two mutations, K679X and IVS5-11 T>G, were present in more than one sample. Each technique was consistent in regards to either detecting the mutation or not detecting it when present. No technique identified false positives for the cell lines without truncating mutations, and all techniques identified the 3875del4, 4600delAA, 5382insC, 185delAG, 3790ins4, 2594delC, Q1313X, 3829delT, and 1294del40 mutations. There were differences in the sensitivities of the various techniques to detect the other mutations (Table 2). PTT with 5' sequencing correctly identified all the truncating mutations. Detection of large gene rearrangements like the deletion of exon 22 is not possible with the PCR-based DNA methods, because the primers cannot anneal to the mutant strand when the DNA is absent. Thus only the wild-type allele

TABLE 2. Validation for Truncating Mutations in *BRCA1*

Sample	Truncating mutations	DHPLC	Sequencing	EMD	TDGS	PTT	PTT+ ^a	SSCP
UT1	3875del4	✓	✓	✓	✓	✓	✓	✓
UT2	IVS5-11T>G	✓	✓	✓	✓	Not detected	✓	Not detected
UT3	4600delAA	✓	✓	✓	✓	✓	✓	✓
UT4	IVS5-11T>G	✓	✓	✓	✓	Not detected	✓	Not detected
UT5	5382insC	✓	✓	✓	✓	✓	✓	✓
UT6	IVS-5-12 A-G	✓	✓	✓	✓	Not detected	✓	Not detected
UT7	185delAG	✓	✓	✓	✓	Not detected	✓	✓
UT8	K679X	✓	✓	✓	✓	✓	✓	Not detected
UT9	3790ins4	✓	✓	✓	✓	✓	✓	✓
UT10	2594delC	✓	✓	Detected ^b	✓	✓	✓	✓
UT11	Exon22 del	Not detected	✓ ^c	Not detected	Not detected	✓	✓	Not detected
UT12	Q1313X	✓	✓	✓	✓	✓	✓	✓
UT13	3829delT	✓	✓	✓	✓	✓	✓	✓
UT14	2985del5	✓	✓	✓	Not detected	✓	✓	✓
UT16	None	✓	✓	✓	✓	✓	✓	✓
UT17	None	✓	✓	✓	✓	✓	✓	✓
UT18	Y1463X	✓	✓	✓	Not detected	✓	✓	Not detected
UT19	None	✓	✓	✓	✓	✓	✓	✓
UT20	None	✓	✓	✓	✓	✓	✓	✓
UT21	K679X	✓	✓	✓	✓	✓	✓	Not detected
UT22	1294del40	✓	✓	✓	✓	✓	✓	✓
	Sensitivity ^d	$\frac{14}{15} = .93$	$\frac{14}{15} = .93$	$\frac{14}{15} = .93$	$\frac{12}{15} = .80$	$\frac{12}{15} = .80$	$\frac{15}{15} = 1.0$	$\frac{10}{15} = .67$

^aPTT+ includes sequencing of the 5' end of the gene (specifically exons 2, 3, 5, and 6).

^bDetected on second pass.

^cSince sequencing is not able to detect exonic deletions directly, presence of a Dutch mutation was indicated before analysis.

^dBased on the 15 distinct mutations.

will be amplified in the heterozygote carrier. There were two mutations that were not detected by TDGS and five unique truncating mutations that were missed by SSCP as indicated in Table 2.

Enzymatic Mutation Detection

The EMD technique was able to correctly identify the *BRCA1* genotype in all but the exon 22 deletion sample (UT11). In addition, as an unclassified variant (UV) was detected in the initial screen for one sample (UT10), full gene analysis was not performed by EMD. Later, 2594delC, a truncation mutation, was confirmed upon EMD evaluation of exon 11. The EMD method has the advantage of having high specificity for all types of alterations including insertions, deletions, and base-substitution mismatches.

While many mutation detection assays are available, EMD is simple, specific, and easy to use. EMD has been shown to be 100% sensitive for 81 different homozygous single-base pair mutations in the mouse beta globin promoter [Youil et al., 1996]. The enzyme has been shown to cleave at all possible mispairings, but the efficiency of cleavage varies considerably depending on the mismatch and the local nucleotide sequence [Pottmeyer and Kemper, 1992; Solaro et al., 1993]. It has been suggested that nucleotide-related deviations in the conformation of DNA in one area can influence the conformation of DNA at another [Drew and Travers, 1985]. We, and others [Orway et al., 2000], have found that the EMD is also more efficient for heterozygous mutation screening than is direct sequencing, as some point mutations that are ambiguous in the automated sequencing results are readily detected by EMD. The test conveniently allows for the detection of multiple sequence variants in the same PCR product reaction, even those separated by only several base pairs. Relatively large fragments may be analyzed as well (>1,000 bp). The technology is thus particularly well suited to the detection of mutations in large genes frequently mutated at unpredictable locations. Multiplexing can be performed to further reduce the overall cost of screening large genes such as *BRCA1* and *BRCA2* [Kulinski et al., 2000; Oleykowski et al., 1998].

TDGS

Recent studies have established the utility of TDGS for gene mutational scanning [Dhanda et al., 1998a,b; Li and Vijg, 1996; Rines et al., 1998; Van Orsouw and Vijg, 1999; Van Orsouw et al., 1996, 1998, 1999]. TDGS is based on parallel rather than serial processing of the mutational target fragments, i.e., by using a combination of extensive multiplex PCR amplifications and two-dimensional DNA electrophoresis in denaturing gradient gels. Mutational variants are detected on the basis of an aberrant

melting behavior, similar to DHPLC (see below). Advantages of TDGS include 1) simultaneous localization of mutations to specific exons of the entire *BRCA1* gene in one single assay on the basis of DNA fragment size and melting temperature characteristics, and 2) the possibility to recognize recurrent mutations or polymorphisms on the basis of unique 4-spot configurations, which obviates the need for sequence confirmation of all variants detected. These features of TDGS greatly increase test sensitivity, reduce human labor, and permit screening of large numbers of samples at relatively low cost [Van Orsouw et al., 1999]. TDGS is capable of detecting point mutations, as well as small insertions and deletions that are either in-frame or frameshifts [Dhanda et al., 1998a,b; Li and Vijg, 1996; Rines et al., 1998; Van Orsouw and Vijg, 1999; Van Orsouw et al., 1996, 1998, 1999]. As expected, the TDGS did not identify the large exon 22 deletion. In addition, the 2985del5 and Y1463X mutations were initially missed due to design flaws. Indeed, the small deletion was initially overlooked because its heteroduplex molecules were so greatly retarded in the size separation part of the 2-D electrophoresis that they fell outside the scanned region (a case where the extra size separation was obviously not advantageous). By enlarging the scanning region the mutation was detected the second time around. The Y1463X mutation was initially missed due to suboptimal primer design, but could be detected after the primers for exon 14 were redesigned and optimized.

Protein Truncation Test

Since mutations in the 5' region of the gene result in protein products that are too small to be readily detectable by PTT alone [Özçelik et al., 1996], one laboratory used PTT with complementary sequencing of exons 2, 3, 5, and 6. PTT with 5' complementary sequencing identified all of the 15 truncating mutations in this panel. As indicated in Table 2, three (20%) of the 15 mutations were missed in the absence of complementary sequencing of the 5'-end. Complete PTT analysis of *BRCA1* requires the use of both RNA and DNA as a template. Exon 11 of *BRCA1* covers approximately 60% of the coding region and eight out of 12 mutations (66.6%) detected by PTT were in this region. The remaining exons were analyzed using RNA as a template, where four out of 12 truncating mutations were detected.

PTT has several advantages over other conventional mutation detection methods. It allows the analysis of large regions of coding sequences ranging from 1 to 3 kb of sequence, compared to approximately 200 to 300 bp regions covered by many other conventional methods. In addition to coding region mutations, PTT can also detect protein truncating mutations that are present outside the coding region. These mutations include internal intronic sequence

changes, which cause cryptic splice sites resulting in splice errors. Alterations resulting from large genomic deletions or insertions also represent a mechanism of mutations in *BRCA1*. These changes usually result in the exclusion of entire exon(s), causing frameshift and consequently premature termination. The exon 22 deletion mutation in case UT11 in this study is an example (see Table 2). Although the genomic sequence alteration causing the exon 22 deletion requires confirmation with additional methods, PTT was able to detect this deletion at the mRNA level. Additionally, PTT can be utilized to investigate the effect on protein product of subtle, previously unclassified alterations in *BRCA1*. To this end, we have recently found that a previously unclassified base substitution in *BRCA1* (4603G-T) leads to a truncated protein product as a result of aberrant splicing [Özçelik et al., 1999]. In addition, PTT with 5' complementary sequencing of *BRCA1* is able to detect Cys61Gly in exon 5 (H.O., unpublished observation). Cys61Gly is one of the most common missense mutations that has been shown to associate with disease [Gorski et al., 2000] and to have a functional consequence. Thus, PTT combined with 5' sequencing is a valuable approach to efficiently detect deleterious mutations in *BRCA1* that may be missed by other mutational detection methodology, including direct DNA sequencing.

The primary limitation of PTT is that it does not detect single-base pair substitutions (missense, polymorphisms, and unclassified variants) that do not result in truncation of the protein. However, since it is unknown whether most of these changes affect the function of the protein, PTT is a clinically feasible method to detect deleterious mutations. The sensitivity of the PTT method in detecting deleterious protein truncating mutations is high, although this may be influenced by its design and application. For example, there is a decreased sensitivity in detecting mutations within the first few hundred bp of the designed PTT fragment. This is partially due to the instability of the shorter truncated proteins and to insensitivity of the gel systems used in detecting these short proteins. However, these problems can be overcome by increasing the overlapping regions between the PTT fragments, as well as developing more sensitive gel systems.

In this validation study, PTT with the complementary 5'-sequencing detected all of the truncating mutations in *BRCA1*, strongly suggesting its usefulness as an efficient mutation detection tool. An additional method could be used to screen for mutations in domains where missense mutations appear to disrupt function. This study also suggests the application of this strategy to other large multi-exonic genes where the majority of deleterious mutations result in protein truncations.

Single Strand Conformation Polymorphism Analysis

Using SSCP, the first lab identified eight of the 15 mutations and the second lab identified 10 of the mutations. The second lab detected all nine of the small frameshift mutations. The large deletion was not detected because primers would have been within the deletion and thus there was no amplification of the deleted fragment. For the three nonsense mutations, the first lab did not detect them and the second lab was only able to detect the Q1313X, and obtained identical false negatives for both samples with the K679X. Both laboratories were also unable to detect the two splice variants, which were base substitutions one base pair apart, IVS5-11 and IVS5-12. The sensitivity of this method was lower than expected at 66% (10/15 mutations). The variation between the two laboratories may have been due to differences in placement of primers such that the second lab was able to detect some mutations more readily. The K679X variant was able to be detected by the second laboratory when the electrophoresis time was decreased; otherwise the variant band was run off the gel. The Y1463X mutation could be detected using a 5% glycerol gel. Even with redesign of primers we were unable to detect the two splice mutations.

SSCP is one of several methods that are able to detect nucleotide changes by relying on the observation that segments of DNA containing alterations such as insertions, deletions, or even single base substitutions, exhibit mobility shifts in polyacrylamide gels when compared to their wild-type counterparts. It is a technique that examines changes that occur in the folding pattern of single stranded DNA. The advantages of SSCP over other methods is that it allows an investigator to pre-screen a large number of samples at a reduced cost, using the same general methodologies and equipment as for genotyping. The screen is then followed by sequencing a limited subset of fragments exhibiting an aberrant mobility.

The disadvantage of SSCP is that it is not as sensitive as other methodologies to detect all types of mutations. Experiments on SSCP sensitivity indicate a range of 63 to 95%, and is highest for detection of frameshift mutations that involve length differences in addition to base alterations [Vidal-Puig and Moller, 1994; Jordanova et al., 1997]. This is noteworthy in regard to *BRCA* analysis, since most reported sequence variants are frameshift in nature [Breast Cancer Information Core, 2001]. The optimization of PCR primers, reaction conditions, and electrophoresis conditions for all exons of these genes further improves detection sensitivity. However, SSCP will not detect large deletions. Given the lower sensitivity of SSCP compared to the other methodologies, it is likely that the prevalence of *BRCA1* mutations is higher than that reported in studies where SSCP was

the screening method. SSCP is not an appropriate choice for clinical testing for *BRCA1*.

Denaturing High Performance Liquid Chromatography (DHPLC)

DHPLC is a detection method based on heteroduplex formation [Xiao and Oefner, 2001]. Detection is carried out after PCR amplification of DNA fragments of interest; these fragments are denatured and then slowly cooled, allowing hybridization of the DNA fragments. In samples that are heterozygous for single-nucleotide substitutions, small deletions or insertions hybridize to produce a mixture of hetero- and homoduplexes. These fragments are then resolved differently after passing through a DNA separation column in a DHPLC machine. Samples showing a different pattern than known homozygous wild-type samples or samples with multiple peaks on DHPLC analysis are assumed to contain sequence variants and then undergo sequence analysis.

The advantage of this system is that after PCR setup and amplification, little further processing is needed; samples are placed into the machine and automatically loaded into the column. If any abnormal fragments are discovered, the remaining aliquot from that sample can then be used for sequencing. DHPLC analysis is capable of detecting single base substitutions, small insertions, and deletions and is a good screening method. This is a major advantage over other systems that may be more discriminating in the types of changes that they detect. A disadvantage of the DHPLC method is that because it does not detect every single nucleotide change, for common polymorphisms, the majority of samples will then need to undergo sequence analysis. For this reason, PCR primers designed to exclude intronic polymorphisms that are not of interest may be necessary to reduce the number of samples that need to be sequenced. However, this is fragment-specific in that many fragments may not contain common variants.

CONCLUSION

As described in this validation study, each method for detection of *BRCA1* truncating mutations has advantages and disadvantages. Before initiating this validation study, we had considered DNA sequencing by Myriad Genetic Laboratories, Inc., as the optimum method. However, DNA sequencing and the other DNA-based methods do not detect large deletions. PTT has the advantage that large deletions can be identified, if the deleted exons are within the region amplified by the primers; on the other hand it has the disadvantage that it can not detect missense mutations. While most documented deleterious mutations in *BRCA1* and *BRCA2* are truncation alterations, several missense changes have been associated with disease (e.g., the *BRCA1* C61G [Gorski et al., 2000]

and M1775R [Miki et al., 1996] mutations). The clinical significance of other missense variants may be proven by investigating their segregation in multiple affected families [Barker et al., 1996; Janezic et al., 1999; Jin et al., 2000; O'Brien et al., 1999; Vega et al., 2001]. Crystal structure predictions for the *BRCA1* protein indicate several published missense changes to be located on the protein surface or at a putative interface with an interacting protein. These missense positions may be critical for proper scaffolding and/or *BRCA1* protein function or other biological changes [Daly et al., 1990; Ito et al., 1993]. It is necessary for a clinical test to be able to detect truncating frameshift and nonsense mutations, large deletions, splice variants, as well as missense mutations. Thus, we believe that it will be important to continue pursuit of other analytic methods or a combination of techniques with high accuracy and lower costs, particularly when very large numbers of samples are studied.

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