Technical Advance

A Rapid and Reliable Test for *BRCA1* and *BRCA2* Founder Mutation Analysis in Paraffin Tissue Using Pyrosequencing

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The founder mutations in *BRCA* (*BRCA1185delAG, *BRCA1**5382insC, and *BRCA2**6174delT) account for 95% of the detectable *BRCA* mutations in breast and ovarian cancer families of Ashkenazi Jewish ancestry. Optimal clinical management of individuals from these high-risk families relies on the identification of *BRCA* founder mutations in the laboratory. We have therefore developed a rapid and reliable approach using pyrosequencing, which allows for the detection of these frequent frameshift mutations on different types of specimens. We were able to correctly identify all mutants in a blinded analysis of 177 DNA samples, including 120 DNA samples extracted from paraffin tissues, 30 samples derived from blood specimens, and 27 samples derived from saliva. The mutation detection rate of pyrosequencing was 100% for all of the DNA samples tested with neither false-positive nor false-negative results. The assay also demonstrated both high accuracy and high precision for the detection of these common mutations in paraffin tissues. Furthermore, saliva collection is a noninvasive alternative for DNA isolation in both clinical and research settings. We show that pyrosequencing is a rapid and reliable method that serves as an excellent platform for *BRCA* founder mutation analysis, especially when only paraffin-embedded tissues are available. (*J Mol Diagn* 2009, 11:176–181; DOI: 10.2353/jmoldx.2009.080137)**

Germline mutations in *BRCA1* and *BRCA2* confer a high risk of breast and ovarian cancer. The estimated lifetime risk of breast cancer and ovarian cancer ranges from 60 to 85% and 15 to 65%, respectively.^{1–3} In the Ashkenazi Jewish population, the combined frequency of mutations in *BRCA1* and *BRCA2* (*BRCA1**185delAG, *BRCA1**5382insC, and

*BRCA2**6174delT) exceeds 2%. These founder mutations account for ~95% of detectable *BRCA* mutations found in dominantly inherited early-onset breast and ovarian cancer families from Ashkenazi Jewish ancestry.^{3–5} Strategies for risk reduction include increased surveillance, preventive surgery, and chemoprevention; for example, salpingo-oophorectomy in carriers of *BRCA* mutations can decrease the risk of breast cancer and *BRCA*-related gynecological cancer.⁶ The information of *BRCA* founder genotyping is also important for perioperative decisions for Ashkenazi Jewish breast cancer patients.

A major limitation in counseling unaffected women from families with inherited breast and ovarian cancer is that the wild-type sequence of *BRCA1* and *BRCA2* is of limited clinical utility because these women may have mutations in other breast cancer susceptibility genes.^{7,8} A true-negative interpretation of wild-type *BRCA* result of the proband cannot be inferred in the absence of demonstration of a *BRCA* mutation segregating in the kindred. Analysis of DNA derived from archival pathology material of deceased affected individuals in such kindreds offers the promise of clarifying these uninformative negative interpretations for unaffected probands, thus mitigating the need for intensified surveillance and/or risk-reducing surgery.

Another limitation of clinical use of *BRCA* testing relates to the timeliness of and accessibility to testing for women newly diagnosed with breast cancer. These women may alter their primary treatment choices based on germline DNA information. Women with *BRCA* mutations may, for example, elect bilateral mastectomy rather than lumpectomy. To make testing more widely available, the turn-around time for the test needs to be rapid. In

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addition, to improve uptake of testing, it would be useful to use saliva instead of blood obtained by needlestick as a source of constitutional DNA. Pyrosequencing of saliva offers an approach to address these clinical needs.

Current approaches used for *BRCA* founder mutation analysis include direct DNA sequencing,⁹ restriction enzyme digestion,¹⁰ and denaturing polyacrylamide gel electrophoresis.^{11,12} However, the direct DNA sequencing approach may not work well with degraded DNA extracted from paraffin-embedded tissues. It was reported that the detection rate was dependent on the age of the blocks being used and only a 45 to 55% rate of correct identification of frameshift (deletion and insertion) mutations was obtained using different aged blocks and the direct DNA sequencing approach.⁹ The multiplex polymerase chain reaction (PCR) and denaturing polyacrylamide gel electrophoresis approach is quick but it cannot substitute for sequence analysis, because non-founder *BRCA1* and *BRCA2* mutations may still occur in these genetic isolates.^{11,12} For the digestion approach, other mutations that alter the restriction enzyme recognition sites may cause false-positive or false-negative results. To address these issues, we developed a new rapid and reliable mutation detection method using pyrosequencing.

Pyrosequencing is a sequencing-by-synthesis-based method. Pyrosequencing is real-time, nonelectrophoretic, nucleotide extension sequencing for various applications,^{13,14} including single nucleotide polymorphism genotyping,¹⁵ bacterial strain typing,¹⁶ mutation detection in tumors,^{17,18} and quantitative CpG island methylation analyses.^{19–21} The methodology is simple, robust, and sensitive, with a detection limit of ~5% mutant alleles, thus, it is particularly useful for tumors containing abundant nonneoplastic cells.²² We have proved pyrosequencing is a rapid and reliable method for *BRCA* founder mutation analysis. Because saliva collection is a noninvasive alternative for DNA isolation in clinical and research settings, this approach is especially attractive for maximizing the participation rate in population genetic studies as well as dealing with some clinical situations in which patients and/or their relatives are not available for on-site whole blood collection.

Materials and Methods

Materials

The 120 DNA samples extracted from formalin-fixed, paraffin-embedded tissues were described and tested previously using denaturing polyacrylamide gel electrophoresis.¹¹ These 120 DNA samples were screened using the pyrosequencing approach described below.

DNA was extracted from 30 peripheral blood specimens and 27 saliva specimens from individuals who received care at the Clinical Genetics Service Clinic at Memorial Sloan-Kettering Cancer Center from February to May 2002. These 57 DNA samples were tested using both direct DNA sequencing and pyrosequencing approaches.

DNA Extraction from Saliva

DNA was extracted from saliva using the following procedures modified from the manufacturer's protocol (Oragene DNA self-collection kit; DNA Genotek Inc., Ottawa, Canada). Briefly, the Oragene saliva sample was incubated in the Oragene vial at 50°C in a water bath for a minimum of 1 hour (the sample may be incubated overnight if this is more convenient). This incubation step only needs to be performed once). The 500- μ l Oragene saliva sample was transferred to a 1.5-ml microcentrifuge tube. Twenty μ l (1/25th volume) of Oragene Purifier (supplied with kit) was added and mixed gently by inversion. The sample was incubated on ice for 10 minutes and centrifuged for 3 minutes at 15,000 $\times$ *g* (13,000 rpm) at room temperature. The pellet was discarded. An equal volume of room temperature 95% ethanol was added to the supernatant, mixed gently by inversion, and incubated for 10 minutes at room temperature to precipitate the DNA. The sample was centrifuged for 1 minute at 15,000 $\times$ *g* (13,000 rpm) at room temperature and the supernatant was discarded without disturbing the pellet. The DNA pellet was resuspended in 100 μ l of DNA hydration solution (Qiagen, Valencia, CA). To fully rehydrate the DNA, the tubes were vortexed followed by incubation overnight at room temperature. Alternatively, incubation for 10 minutes at 65°C is also effective.

DNA Amplification and Mutation Analysis by Direct DNA Sequencing

DNA from case samples was analyzed for the three common Ashkenazi founder mutations in *BRCA1* and *BRCA2* (185delAG and 5182insC in *BRCA1* and 6174delT in *BRCA2*) using the following oligonucleotide primers flanking the mutation loci: *BRCA1*, 185delAG forward (5'-GAAGTTGTCATTTTATAAACCTTT-3') and 185delAG reverse (5'-TGTCTTTTCTTCCTAGTATGT-3') and 5382insC forward (5'-ATATGACGTGTCTGCTCCACT-3') and 5382insC reverse (5'-AATGAAGCGGCCCATCTC-3'); and *BRCA2*, 6174delT forward (5'-CTAAATATCACCT TGTGATGTTAG-3') and 6174delT reverse (5'-GTGAGCTGGTCTGAATGTTTCGTTAC-3'). The PCR product was purified and subjected to DNA sequencing by Big Dye Terminator cycle sequencing (Applied Biosystems, Foster City, CA) with the primers used for PCR amplification. The sequencing product was separated by capillary electrophoresis on an ABI 3730 DNA analyzer (Applied Biosystems). The sequences were compared with the reference sequences.

DNA Amplification and Mutation Analysis by Pyrosequencing

Primers used for PCR amplification and pyrosequencing are summarized in Table 1. Each PCR reaction contained the forward and reverse primers (final concentration of 0.2 μ mol/L), 0.125 mmol/L of each dNTP, 1.5 mmol/L MgCl₂, 1 $\times$ PCR buffer, 1.5 U of Taq polymerase (HotStar Taq kit, catalog no. 203443; Qiagen), and 20 to 50 ng of template DNA in a total volume of 50 μ l. PCR conditions

Table 1. The PCR Primers and Sequencing Primers used for *BRCA1* Founder Mutation Analysis by Pyrosequencing

Mutation	Primer name	Primer sequence*	Product size (bp)
<i>BRCA1*185delAG</i>	185delAG-F (biotinylated)	5'-CGCGTTGAAGAAGTACAAAATGT-3'	80
	185delAG-R	5'-ACTCTTGTGCTGACTTACCAGATG-3'	
	delAG sequencing primer	5'-TGACTTACCAGATGGGA-3'	
<i>BRCA1*5382insC</i>	5382insC-F	5'-AAAGCGAGCAAGAGAATCCC-3'	72
	5382insC (biotinylated)	5'-TGGGGTGAGATTTTGTCAAC-3'	
	InsC sequencing primer	5'-CGAGCAAGAGAATCCC-3'	
<i>BRCA2*6174delT</i>	6174delT-F	5'-GTCTCATCTGCAAATACTTGTGG-3'	86
	6174 delT-R (biotinylated)	5'-CTTGCCTTTGTATGAAGCAT-3'	
	delT sequencing primer	5'-GGGATTTTTCAGCACAGC-3'	

*The GenBank references for primer sequences are: *BRCA1* (NM 007304) and *BRCA2* (NM 000059).

consisted of initial denaturing at 94°C for 15 minutes; 45 cycles of 94°C for 20 seconds, 55°C for 20 seconds, and 72°C for 30 seconds; and a final extension at 72°C for 7 minutes. The PCR products were separated by agarose gel electrophoresis to confirm successful amplification. The PCR products (each 10 μ l) were sequenced by pyrosequencing on a PSQ96 HS system (Biotage AB, Uppsala, Sweden) following the manufacturer's instructions using all three pyrosequencing primers (Table 1). The PCR primers and sequencing primers are indicated in Figure 1. Sequences to be analyzed and nucleotide dispensation order are shown in Figure 2 under each pyrogram.

Negative control DNA was extracted from a patient who does not have *BRCA* mutations. Positive control DNA was extracted from blood obtained from *BRCA* mutation carriers. Both control DNAs were tested by restriction enzyme digestion and confirmed by direct DNA sequencing. Negative control, positive control, and blank tube without DNA (reagent contamination control) were included in each run.

Results

Design and Interpretation of the Pyrosequencing Assay

The pyrosequencing assay was designed to begin sequence analysis right at the mutation site (*BRCA1*

**5382insC*), two nucleotides 3' of the mutation site (*BRCA1*185delAG*), or three nucleotides 5' of the mutation site (*BRCA2*6174delT*) (Figure 1). A sequential nucleotide dispensation protocol was used that reflects the expected order of nucleotide incorporation and the potential base changes (Figure 2). In addition, negative nucleotide dispensations were inserted to serve as internal controls for nucleotide misincorporation. Peak heights are proportional to the number of nucleotides that are incorporated with each dispensation (Figure 2).

For the wild-type sequence for *BRCA1*185delAG* (Figure 1A and Figure 2A), the generated peak heights for nucleotide dispensations at positions 2, 3, 4, 5, 6, and 7 (CACTCT in the reverse strand) are approximately equivalent and represent incorporation of a single nucleotide in both alleles (2 light units). Note that the signal intensity of the A at position 9 is two times greater (4 light units) and reflects the incorporation of two As in tandem in both alleles (four As in total). Using the peaks at positions 2, 3, 4, 5, 6, or 7 as an internal reference, a peak equivalent to 1 light unit within the variable site represents the incorporation of a nucleotide into only one of two alleles and the detection of a heterozygous deletion mutant. The intensities of C and T peaks at positions 6 and 7 are approximately half of those in the wild-type control (1 light unit), which indicates a deletion of CT in the reverse strand in one allele (heterozygous delAG in the forward strand). For *BRCA1*5382insC* (Figure 1B and Figure 2B), incorporation of an extra C was seen in the mutant, which is absent in the wild-type control. Because the peak intensity of the C (1 light unit) is approximately half of other peaks at positions 4, 6, 7, and 8, it represents an insC in only one of the alleles (heterozygous insC).

For the wild-type *BRCA2*6174delT* (Figure 1C and Figure 2C), we saw an incorporation of 4 unit peak for A at position 2 (two As in tandem in both alleles, four As in total), 2 unit peak for G and T at positions 3 and 4, and 4 unit peak at position 5 (two Gs in tandem in both alleles, four Gs in total). The very high peak of 8 light units at position 7 represents four As in tandem in both alleles (eight As in total). For the heterozygous mutant *BRCA2*6174delT*, the incorporation of 4 light units of As is the same as in wild type. However, because the T after G is deleted in the mutant allele, all of the three Gs in tandem are incorporated. Therefore, the peak at position 2 represents 4 units of G in both alleles (one G from the wild-type allele and three Gs from the mutant allele).

Figure 1. Assay design for *BRCA1* and *BRCA2* Ashkenazi Jewish founder mutation analysis using pyrosequencing. *BRCA1*185delAG* (A), *BRCA1*5382insC* (B), and *BRCA2*6174delT* (C) were amplified using the PCR primers indicated. The pyrosequencing primers are shown by dotted lines as they overlap with PCR primers. Nucleotide positions of the founder mutations are boxed and in bold.

Figure 2. Representative pyrograms by pyrosequencing. Sequence to be analyzed and dispensation order used for *BRCA* founder mutation analysis were shown under each pyrogram. The specific mutations were shown in bold in brackets. **A:** Pyrograms for wild type and *BRCA1**185delAG. Heterozygous deletion of AG (arrows) causes a decrease by half at CT in reverse strand (AG in forward strand). The 2 units G (arrowheads) at pyrogram positions 1 and 8 are negative nucleotide dispensations that serve as internal controls for nucleotide misincorporation. **B:** Pyrograms for wild type and *BRCA1**5382insC. Heterozygous insertion of C indicated by an arrow is present in the mutant, but not in the wild-type sample. One unit G and one unit T (arrowheads) at pyrogram positions 1 and 3 are negative nucleotide dispensations that serve as internal controls for nucleotide misincorporation. **C:** Pyrograms for wild type and *BRCA2**6174delT. For the heterozygous *BRCA2**6174delT, the peak at position 2 represents 4 units of G in both alleles (one G from the wild-type allele and three Gs from the mutant allele). Because the T is deleted in the mutant allele, the T in the wild-type allele gives 1 unit light (T at position 4). After the incorporation of T, the next two Gs in the wild-type allele were extended so the G peak is approximately half of that in wild-type sequence (with four Gs). Two light units of C (arrowheads) at pyrogram positions 1 and 6 are negative nucleotide dispensations that serve as internal controls for nucleotide misincorporation.

Because the T is deleted in the mutant allele, the T in the wild-type allele gives 1 unit light (T at position 4). After the incorporation of T, the next two Gs in the wild-type allele were extended so the G peak is approximately half of that in wild-type sequence (with four Gs). The intensity of the A peak, which is not affected by the delT mutation, is equal in both wild-type and mutant samples.

Accuracy and Precision

The laboratory investigators were blinded to the lymphocyte and paraffin tissue-derived DNA genotyping results. Results of the archival DNA analysis using pyrosequencing were sent to the study coordinator. The sample codes were disclosed and the study coordinator compared the results from the coded samples with the original results obtained using lymphocyte and paraffin tissue DNA. Because we designed PCR primers that generate relative small PCR products (Table 1), we were able to obtain satisfactory PCR fragments for all of the 120 paraffin-

extracted DNA samples (27 cases were negative and 93 cases were positive for one of the three *BRCA* mutations). There was 100% concordance on results using pyrosequencing and denaturing polyacrylamide gel electrophoresis. There were no false-negatives or false-positives. The age of the different blocks did not interfere with the results. All mutant genotypes were confirmed by repeat analysis; all samples with a mutation showed a very clear distinct pyrogram specific for that mutation, which is identical to that of the positive control included in each run (Figure 1).

For the blood and saliva specimens, mutation analysis was performed in parallel using both pyrosequencing and direct DNA sequencing analysis. We obtained concordant results on all of the 57 samples tested using these two different approaches. For the 30 blood DNA samples, 5 cases were negative and 25 cases were positive for one of the three *BRCA* mutations. For the 27 saliva DNA samples, 8 cases were negative and 19 cases were positive for one of the three *BRCA* mutations.

Cost Analysis

The pyrosequencing assay is relatively simple and cost effective compared with other approaches such as direct DNA sequencing. Whereas dideoxy (BigDye Terminator) sequencing requires lengthy personnel time on purification of PCR products, setting up sequencing reactions, purification of postsequencing reactions, which takes several hours, the simple purification step with pyrosequencing after PCR only takes 5 to 10 minutes. For a full 96-well plate, the results are obtained in 15 minutes after the plate is put into the pyrosequencer whereas it may take 2 hours for an ABI 3730 DNA analyzer. Because pyrosequencing approach involves many fewer steps after PCR, it uses fewer reagents as well. A rough estimate of the total reagent cost of such testing is probably less than US \$10 per sample, which includes reagents for DNA extraction from paraffin tissue, peripheral blood or saliva, PCR reagents, and pyrosequencing reagents. Thus, pyrosequencing may be more cost effective than direct DNA sequencing in terms of both reagents and labor time.

Discussion

In this study, we tested 120 formalin-fixed, paraffin-embedded tissue samples, including 25 samples older than 10 years, for any of the three *BRCA* Ashkenazi founder mutations. We were able to obtain equally satisfactory results on all of the samples tested. No difference was found in the quality and reproducibility of the results between old and new samples. The success of our method can mainly be attributed to the small size of the amplicons (72 to 86 bp), the high-fidelity Taq polymerase used, and most importantly the reliable and sensitive mutation detection approach we chose.

Pyrosequencing has a number of advantages over traditional methods for genotyping. The technique offers high accuracy, flexibility, automation, and parallel processing (96 samples at one time within 20 minutes). Pyrosequencing does not require electrophoresis, size separation, labeled nucleotides, or labeled primers (except biotinylated PCR primer). We have checked the pyrosequencing results by direct DNA sequencing in 57 of the DNA samples and found they were in 100% agreement.

Pyrosequencing has been widely used for characterization of cancer mutations, quantification of CpG methylation at multiple CpG sites,^{19–21} sensitive detection of tumor cells among normal cells,^{22–24} and quantification of genetic alterations such as loss of heterozygosity. It proved to provide a substantial advantage in mutation detection rates compared with dideoxy sequencing, as demonstrated in the studies on detection of *KRAS* mutations from DNA mixtures of paraffin-embedded colon cancer and normal tissue as well as from paraffin-embedded pancreatic cancers.²²

Paraffin-based *BRCA* testing of archival material may have a significant clinical impact. We have identified as many as 1000 unaffected women from high-risk breast

families, who have tested negative for *BRCA* mutations. These women have deceased family members whose DNA could possibly be retrieved for archival DNA testing. We have implemented this strategy in our clinic and started to test these families as a standard clinical test.

It is known that sequence alterations can occur because of formalin fixation of archival specimens. It has also been found that very low amounts of template DNA can magnify the detection of rare PCR errors.^{25–28} Given that the majority of artificial sequence alterations are substitution mutations and only 4 to 5% are deletion/insertion mutations^{27,28} and the relatively low frequency of such sequence alterations (up to 1 mutation artifact per 500 bases),²⁷ we expect that the probability of having an artificial insertion or deletion in short PCR fragments (72 to 86 bp) is very low. For clinical testing, we always test patient specimens in duplicates. The chance of having such artificial insertion or deletion in both replicates at the specific mutation sites is even lower.

The quality of paraffin-embedded material can vary with respect to partial or substantial degradation of DNA. This, in turn, affects PCR yields, especially on fragments >300 bp. Therefore, the advantage of designing small PCR fragments (<100 bp) as in the current study, can significantly overcome this limitation.

False-negative results attributable to preferential amplification of the wild-type allele may occur if during PCR only one allele is amplified and not the other. This phenomenon, also called as preferential amplification or allele dropout, is a known limit in amplifying the very small amounts of template DNA that are often extracted from small paraffin-embedded biopsies. Because we routinely use 20 to 50 ng of genomic DNA in PCR reactions, we did not observe this phenomenon in any of the 120 paraffin-extracted DNA samples tested in this study.

In addition to the 120 DNA samples extracted from paraffin-embedded tissues, we were also able to correctly identify all mutants in a blinded analysis of an additional 57 DNA samples, including 30 derived from blood specimens and 27 derived from sputum. We obtained concordant results on all of the 57 DNAs tested using both pyrosequencing and direct DNA sequencing analysis. Furthermore, saliva collection (Oragene DNA self-collection kit) is a noninvasive alternative for DNA isolation in clinical and research settings. This approach is especially attractive for maximizing the participation rate in population genetic studies as well as dealing with some clinical situations in which patients and/or their relatives are not available for on-site whole blood collection. We have also adopted this test to provide rapid turn around (1 week) results to women newly diagnosed with breast cancer who have used this information to inform surgical decisions. Saliva has served as a source of DNA as a confirmation sample for these women, to eliminate the chance of a sample mix-up. However, in the future, a single test from saliva-derived DNA could be sufficient. The applicability of this simple, robust, and sensitive pyrosequencing approach can be used for *BRCA* founder mutation analysis in large-scale studies.

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