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Rapid and Sensitive Detection of *BRCA1/2* Mutations in a Diagnostic Setting: Comparison of Two High-Resolution Melting Platforms

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BACKGROUND: High-resolution melting is an emerging technique for detection of nucleic acid sequence variations. Developments in instrumentation and saturating intercalating dyes have made accurate high-resolution melting analysis possible and created opportunities to use this technology in diagnostic settings. We evaluated 2 high-resolution melting instruments for screening *BRCA1* and *BRCA2* mutations.

METHODS: To cover the complete coding region and splice sites, we designed 112 PCR amplicons (136–435 bp), amplifiable with a single PCR program. LCGreen® Plus was used as the intercalating dye. High-resolution melting analysis was performed on the 96-well Lightscanner™ (Idaho Technology Inc.) and the 96-well LightCycler® 480 (Roche) instruments. We evaluated sensitivity by analyzing 212 positive controls scattered over almost all amplicons and specificity by blind screening of 22 patients for *BRCA1* and *BRCA2*. In total, we scanned 3521 fragments.

RESULTS: All 212 known heterozygous sequence variants were detected on the Lightscanner by analysis on normal sensitivity setting. On the LightCycler 480, the standard instrument sensitivity setting of 0.3 had to be increased to 0.7 to detect all variants, decreasing the specificity to 95.9% (vs 98.7% for the Lightscanner).

CONCLUSIONS: Previously, we screened *BRCA1/2* by direct sequencing of the large exon 11 and denaturing gel gradient electrophoresis (DGGE) for all other coding exons. Since the introduction of high-resolution melting, our turnaround time has been one third of that with direct sequencing and DGGE, as post-PCR handling is no longer required and the software allows fast analyses. High-resolution melting is a rapid, cost-efficient, sensitive method simple enough to be readily implemented in a diagnostic laboratory.

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Different approaches are used to screen the complete coding region of large genes such as *BRCA1*[2] (MIM 113705) and *BRCA2* (MIM 600185), the 2 major breast cancer susceptibility genes. Most commonly, a prescreening method such as denaturing high-performance liquid chromatography (dHPLC)[3] or denaturing gradient gel electrophoresis (DGGE) is used, followed by sequencing of aberrant fragments. Screening of *BRCA1* and *BRCA2* is arduous because of the complex mutational spectrum and the large size of the genes for which the complete coding sequence needs to be analyzed for an increasing number of patients. Both genes lack mutation hot-spot regions, and different types of mutations, including frameshift, missense, nonsense, and splice site, are found. *BRCA1* comprises 22 coding exons; the 7.8-kb mRNA transcript is translated into a protein of 1863 amino acids *(1)*. *BRCA2* comprises 26 coding exons; the 10-kb mRNA transcript is translated into a protein of 3418 amino acids *(2)*.

Until recently, our mutation detection strategy consisted of direct sequencing of the large exon 11 of both *BRCA1* and *BRCA2* and DGGE for all other coding exons *(3, 4)*. Because we were witnessing an increasing number of patients and aiming at a reduction of costs and workload, we evaluated 2 high-resolution melting instruments for mutation screening of both *BRCA1* and *BRCA2*.

High-resolution melting analysis of nucleic acids depends on the ability to record and evaluate fluorescence intensities as a function of the melting tempera-

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[2] Human genes: *BRCA1*, breast cancer 1, early onset; *BRCA2*, breast cancer 2, early onset.
[3] Nonstandard abbreviations: dHPLC, denaturing high-performance liquid chromatography; DGGE, denaturing gradient gel electrophoresis; dsDNA, double-strand DNA; Tm, melting temperature; SNP, single nucleotide polymorphism.

ture of PCR products. The melting behavior of the PCR products is monitored by plotting the changes in fluorescence that occur by denaturing double-strand DNA (dsDNA) upon heating. Heterozygous DNA samples form heteroduplexes, resulting in a different shape of the melt curve compared with a homozygous reference sample. The altered melting curve shape is a result of the presence of both heteroduplex and homoduplex amplicons in the PCR product, vs only homoduplexes from normal samples. Mutant homozygous samples, in contrast, are detected by a melting temperature (Tm) shift rather than an altered curve shape.

Since the introduction of saturating dsDNA binding dyes like LCGreen® Plus, the sensitivity and specificity of DNA melting analysis has increased substantially. Nonsaturating dyes like SYBR Green possibly allow redistribution of dye molecules from melted regions back into the dsDNA amplicon, resulting in no change in fluorescent signal even in the presence of a heteroduplex *(5*, *6)*. When a saturating fluorescent dye is intercalated in dsDNA, dye jumping during amplicon melting is prevented, allowing theoretical detection of all sequence changes.

In the present study, we evaluated high-resolution melting analysis for *BRCA1* and *BRCA2* and compared the Lightscanner™ (Idaho Technology Inc.) and the LightCycler® 480 (Roche) instruments. To determine sensitivity, we used 212 positive controls scattered over 100 different amplicons. In addition, we performed a blind screening of 22 patients (i.e., 2464 PCR reactions) to determine the specificity of high-resolution melting analysis on the 2 instruments.

Materials and Methods

DNA SAMPLES AND STUDY DESIGN

DNA samples from patients with previously characterized genetic variants were used as positive controls to determine the sensitivity of high-resolution melting. These variants were previously detected with a mixture of other techniques such as DGGE, dHPLC, protein truncation test (PTT), and sequencing. For *BRCA1*, 97 positive control samples were available, scattered over 42 of 45 amplicons designed to cover the complete coding sequence of *BRCA1*. For amplicons 11.1, 11.17, and 15.2, we had no positive controls available. We analyzed 36 deletions (15 of 1 bp, 7 of 2 bp, 2 of 3 bp, 6 of 4 bp, 3 of 5 bp, 2 of 11 bp, and 1 of 62 bp), 12 1-bp insertions (of which 10 were duplications), a combined delTTinsG mutation and delAGinsT mutation, and an insertion of an *Alu* element. Furthermore, we analyzed 29 transitions (15 T/C and 14 A/G) and 17 transversions (3 A/C, 9 G/T, 3 C/G, and 2 T/A).

For *BRCA2*, we tested 115 positive control samples scattered over 58 of 67 amplicons, designed to cover the complete coding sequence of *BRCA2*. No positive controls were available for fragments 4, 11.10, 11.13, 12, 21, 26, 27.1, 27.2, or 27.3. For *BRCA2*, we analyzed 38 deletions (13 of 1 bp, 15 of 2 bp, 9 of 4 bp, and 2 of 7 bp), 7 1-bp insertions (of which 5 were duplications), 46 transitions (19 T/C and 27 A/G), and 24 transversions (8 A/C, 6 G/T, 6 G/C, and 4 A/T). An overview of all positive controls is given in Supplementary Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol54/issue6. As negative controls, we used DNA samples from several healthy individuals.

Also, we blindly screened 22 patients for *BRCA1* and *BRCA2* to determine the specificity of the high-resolution melting technique. We had previously analyzed 11 of the patient samples with direct sequencing of exon 11 of *BRCA1/2* and DGGE for all other exons. We performed a second blind screening of 11 patients in parallel with direct sequencing of all amplicons.

PRIMERS AND PCR OPTIMIZATION

We designed primers for *BRCA1* and *BRCA2* to cover the complete coding region and splice sites encompassing exons 2–24 of *BRCA1* (45 amplicons, 21 of which encompass exon 11) and exons 2–27 of *BRCA2* (67 amplicons, 27 of which encompass exon 11). Primer sequences are available in Supplementary Table 2 in the online Data Supplement. The absence of SNPs in the primers was verified with the help of the Ensembl genomic sequence database. We chose annealing temperatures all around 50 °C and evaluated the specificity of the primers using the University of California Santa Cruz *in silico* PCR program. The amplicon length ranged between 136 and 435 bp, median 238 bp. To simplify the sequencing process afterwards, all primers were fused with universal M13-tails (forward CAC GACGTTGTAAAACGAC and reverse CAGGAAA CAGCTATGACC). The dye-stained DNA template had no interference with the sequencing reactions.

PCR was performed in 25 μL volumes. The amplification mixture included 1.5 mmol/L $MgCl_2$ (Invitrogen), 1× PCR buffer (Invitrogen), 3% DMSO (VWR International), 0.2 μmol/L of both forward and reverse primer, 200 μmol/L of each dNTP, 0.5 U/μL Platinum® *Taq* DNA polymerase (Invitrogen), 0.5× LCGreen Plus, and approximately 100 ng DNA. By adding 3% DMSO, we could use the same master mix for almost all fragments. For 3 amplicons (*BRCA1* 11.14 and *BRCA2* 11.5 and 11.6), we increased DMSO concentrations (to 10%, 5%, and 7%, respectively) to obtain specific PCR products.

All 112 amplicons were amplified using a universal touchdown PCR program. The temperature cycling protocol consisted of an initial denaturation step at 94 °C for 2 min, followed by 12 cycles of denaturation

at 94 °C for 20 s, annealing starting at 58 °C for 20 s (decreasing 1 °C per cycle), and extension at 72 °C for 1 min. This initial PCR reaction was followed by 25 additional cycles of denaturation at 94 °C for 40 s, annealing at 46 °C for 40 s, and extension at 72 °C for 30 s. Final extension was accomplished at 72 °C over 10 min.

After amplification, PCR products were denatured at 95 °C for 5 min and cooled (1.7 °C/s) in a thermocycler block to 25 °C to form heteroduplexes.

MELTING ACQUISITION AND MELTING ANALYSIS

Melting acquisition was performed on the 96-well Lightscanner (Idaho Technology Inc.) and the LightCycler 480 (Roche). According to the manufacturer's instructions, we transferred 10 μL PCR product to 96-well plates suitable for high-resolution melting analysis [Lightscanner, 4Titude plates (BioKé); LightCycler 480, Roche]. To prevent evaporation during heating on the Lightscanner, PCR products were covered with a mineral oil overlay. We used a 10-min centrifugation step instead of the 1-min centrifugation specified in the manual. The longer centrifugation turned out to be helpful in the elimination of air bubbles that rise to the surface during the melting process, disturbing the fluorescent curves. The plates on the Lightscanner were heated from 70 °C to 98 °C at 0.1 °C/s.

For the LightCycler 480, the appropriate 96-well plates (Roche) were covered with the accompanying sealing foils. The applied template for high-resolution melting included first-step heating to 95 °C and a melting program that went from 55 °C to 95 °C.

Melting curve analysis was performed on the Lightscanner with Lightscanner software (version 1.5) and on the LightCycler 480 with the gene-scanning module (version 1.3). Both software programs employ a 3-step analysis: 1) normalization by selecting linear regions before (100% fluorescence) and after (0% fluorescence) the melting transition, 2) temperature shifting by moving the curves along the *x*-axis, facilitating grouping, and 3) use of the Autogroup function. Shape differences were further analyzed by subtracting the curves from a reference curve, generating a difference plot, where fluorescence of all curves is plotted against temperature.

Results

SENSITIVITY

For *BRCA1*, we analyzed with high-resolution melting 97 known heterozygous sequence variants (78 pathogenic mutations, 15 unclassified variants, and 4 polymorphisms) spread over 42 of 45 amplicons on the Lightscanner and the LightCycler 480 instruments. To validate the technique for *BRCA2*, we analyzed 115 known sequence variants (58 pathogenic mutations, 33 unclassified variants, and 24 polymorphisms) spread over 58 of 67 amplicons. The melting curves of the positive controls were compared with those of control individuals. We did not have sequence variants available for 12 amplicons and verified their quality by analyzing 8 wild-type samples. For this part of our study, a total of 1057 fragments were scanned on both instruments.

With the Lightscanner instrument, all 97 known heterozygous *BRCA1* sequence variants were detected by analysis on the normal sensitivity setting. In addition, 15 single nucleotide polymorphisms (SNPs) were detected in the healthy control individuals and confirmed by sequencing. On the LightCycler 480, only 92 of 97 (94.8%) of the *BRCA1*-positive controls were detected with the default sensitivity setting of 0.3. Increasing the sensitivity setting to 0.5 led to 100% detection (97 of 97). The variants undetectable at 0.3 shared no specific features—they had no common positions in the fragments, and deletions as well as insertions, transitions, and transversions were missed (examples are shown in Supplementary Fig. 1 in the online Data Supplement).

On the Lightscanner, all 115 known heterozygous *BRCA2* sequence variants were easily detected, except c.1909 + 22insT. This polymorphism is an insert of a thymine in a row of 12 thymines in intron 10, and the pre- and postmelt values had to be carefully adjusted to make this variant detectable (panels are shown in Supplementary Fig. 1 in the online Data Supplement). On the LightCycler 480, 9 of 115 positive controls were not detected with the default 0.3 sensitivity setting (sensitivity 92%). Analysis of the melting curves on sensitivity setting 0.5 led to 100% detection. Detection of the c.1909 + 22insT variant was again possible only after carefully adjusting the pre- and postmelt parameters.

Hence, we detected all possible heterozygous combinations of bases (A/T, A/C, A/G, C/G, C/T, and G/T) with high-resolution melting on both instruments. Also, deletions and insertions of different sizes (1–63 bp) were easily distinguished from wild-type samples (examples shown in Supplementary Fig. 1 in the online Data Supplement). Frameshift mutations are the most frequently detected mutations in *BRCA1/2*.

As mutations in the *BRCA1* and *BRCA2* genes are autosomal dominant, we tested only a limited number of homozygous (polymorphic) variants on both instruments. When only the standard temperature shifting was used, both instruments could distinguish wild-type sequences from sequences with heterozygous and homozygous SNPs. An example is shown in Fig. 1.

SPECIFICITY

To determine the specificity of high-resolution melting, we performed a blind screening of 22 patients for

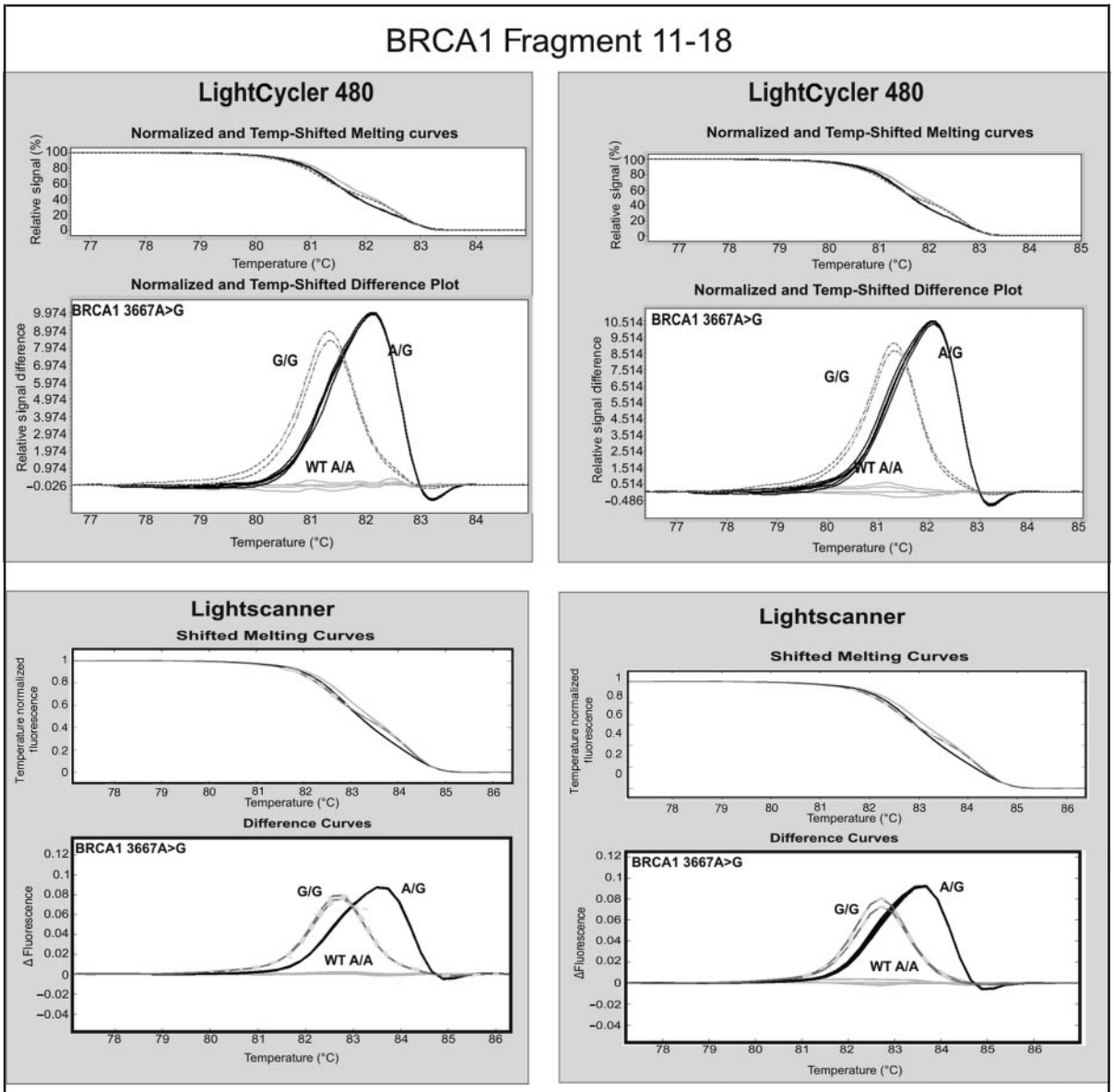

Fig. 1. Detection of heterozygous and homozygous SNPs on both instruments.

Difference plots and melting curves for *BRCA1* fragment 11-8 on the Lightscanner and LightCycler 480. All samples were amplified and analyzed in duplicate. The similarity of the curves for the duplicate samples illustrates the robustness of the conditions applied. SNP *BRCA1* c.3667A>G is shown. Both instruments could easily distinguish the wild-type homozygous (grey baselines) from the heterozygous (black) and homozygous mutant (dotted) melting curves. The melting curves illustrate that mutant homozygous variants are detected by a Tm shift rather than by an altered curve shape. By the use of the default Tm shift setting (that accounts for small temperature variations across the block) on both instruments, most of the homozygous SNPs were detected. There is a 2 °C difference in Tm for these amplicons between the 2 instruments, probably due to internal calibration. Temp, temperature.

BRCA1 and *BRCA2*. We had previously screened 11 patients with sequencing for exon 11 and DGGE for all other coding exons and splice sites of *BRCA1* and *BRCA2*. The other 11 patients were screened with high-resolution melting, and simultaneously all amplicons were sequenced. The analysis was performed on normal sensitivity setting on the Lightscanner and sensitivity settings 0.3, 0.5, and 0.7 on the LightCycler 480.

All sequence variants were detected on the Lightscanner, confirming the 100% sensitivity. However, we observed 32 false positives (confirmed by direct sequencing) in the 2464 amplicons analyzed (22 patients screened for 112 amplicons), i.e., a specificity of 98.7%.

On the LightCycler 480, 87 sequence variations were missed with the standard sensitivity setting of 0.3. A specificity of 98.6% (35 false positives in 2464 fragments) was calculated. As we did not detect all genetic variants without highly adjusting the pre- and postmelt parameters on sensitivity setting 0.5, we reanalyzed our data with sensitivity setting 0.7. This led to a detection of all known genetic variants; however, the specificity dropped slightly to 95.9% (102 false positives of 2464 fragments). An overview of the high-resolution melting specificity per exon is given in Fig. 2.

Discussion

The aim of this study was to evaluate and validate high-resolution melting curve analysis for mutation detection on 2 distinct instruments. The Lightscanner instrument (Idaho Technology) is specially designed for high-resolution melting analysis, and the LightCycler 480 (Roche) was originally launched as a real-time PCR instrument. We used the *BRCA1* and *BRCA2* genes as a model to evaluate the high-throughput capacity of the high-resolution melting technique because these large breast cancer susceptibility genes are being analyzed worldwide in an increasing number of patients.

We designed 112 PCR amplicons, all amplifiable with 1 PCR program, covering the complete coding region of both *BRCA1* and *BRCA2* for high-resolution melting analysis using LCGreen Plus as saturating dye. To accomplish our high-throughput setup, PCR reactions were not performed on the thermocycler present in the LightCycler 480. On both instruments, minimal post-PCR handling is required and analyses can be done very fast.

We evaluated the sensitivity and specificity of high-resolution melting on the 96-well Lightscanner and LightCycler 480 instruments by analysis of 3521 PCR amplicons in total, the largest and most thorough study on both instruments so far. We detected all known sequence variants on both the Lightscanner (normal sensitivity setting) and the LightCycler 480 (sensitivity setting 0.7). For sensitivity setting 0.5 on the LightCycler 480, it was possible to detect all sequence variants only by carefully adjusting the pre- and postmelting parameters. In a diagnostic setting, we recommend analyzing samples on sensitivity setting 0.7 to overcome false negatives.

Previously, 100% sensitivity for high-resolution melting analysis was reported for PCR products up to 300 bp *(7)* or 400 bp *(6)*. This was confirmed by Kennerson et al. *(8)* in a small study on mutation scanning of 4 amplicons of the *GJB1* gene in the 96-well Lightscanner. These investigators also found 100% sensitivity from a validation of high-resolution melting analysis with 18 positive control samples, followed by a blind study of 10 patients. As the new gene scanning software module was only recently made available on the LightCycler 480, we were able to find data from only one other study *(9)* comparing the results of both instruments for factor VIII mutations. The investigators missed 2 of 20 mutations on both instruments using their settings. Because we obtained 100% sensitivity (with the 0.7 sensitivity setting), gene scanning on the LightCycler 480 also seems to be a valuable method.

For specificity, however, the Lightscanner scored slightly better than the LightCycler 480 on a sensitivity setting of 0.7. The decrease of the specificity on the LightCycler 480 was caused by increasing the sensitivity setting from the default value of 0.3 and led to sequencing of about 70 more amplicons than the Lightscanner.

In a recent study, Herrmann et al. *(10)* compared the melting profile of a 110-bp fragment on different instruments. They concluded that the ability to accurately genotype single-base changes by amplicon melting is limited by the spatial temperature variation across the plate, which is lower on the Lightscanner than the on the LightCycle 480. This could explain the somewhat lower specificity found on the LightCycler 480. The specificity of high-resolution melting analysis was also studied by Reed and Wittwer *(7)* with engineered plasmids. They reported 100% specificity for PCR products up to 300 bp, and the specificity was only slightly lowered (99.4%) for larger fragments. Our somewhat lower specificity rate may be due to the use of complex genomic DNA (instead of plasmids) or to the high number of amplicons that we analyzed to screen the complete coding regions of 2 large genes. We also found that PCR conditions need to be well optimized to obtain high values for specificity and sensitivity. After optimization, PCR fragments need to be verified by high-resolution melting curve analysis for several individuals to find out if the curves are reproducible.

We conclude from our data that high-resolution melting analysis is at least as sensitive as other commonly used prescreening methods such as DGGE, dHPLC, or fluorescent conformation-sensitive gel electrophoresis (F-CSGE). Sensitivities and specificities of 100% are reported for these techniques *(4, 11–13)*. However, a recent comparison *(14)* of dHPLC and high-resolution melting found better sensitivity and specificity for the latter. The major advantage of high-resolution melting is the minimal post-PCR require-

Fig. 2. Specificity per exon for *BRCA1* and *BRCA2* by the Lightscanner and the LightCycler 480 with 2 different sensitivity settings.

ment, making it a less labor-intensive method while improving its cost-effectiveness, ease of use, and throughput.

In the present study, we detected several homozygous SNPs by high-resolution melting. We used the standard Tm shift analysis mode (to facilitate grouping). A large study on the detection of homozygous SNPs was performed by Liew et al. *(15)*, who concluded that approximately 4% (class 3 or 4) of homozygous human SNPs will remain undetectable by high-resolution melting due to the small Tm difference generated by homozygous C/G and A/T SNPs. The ho-

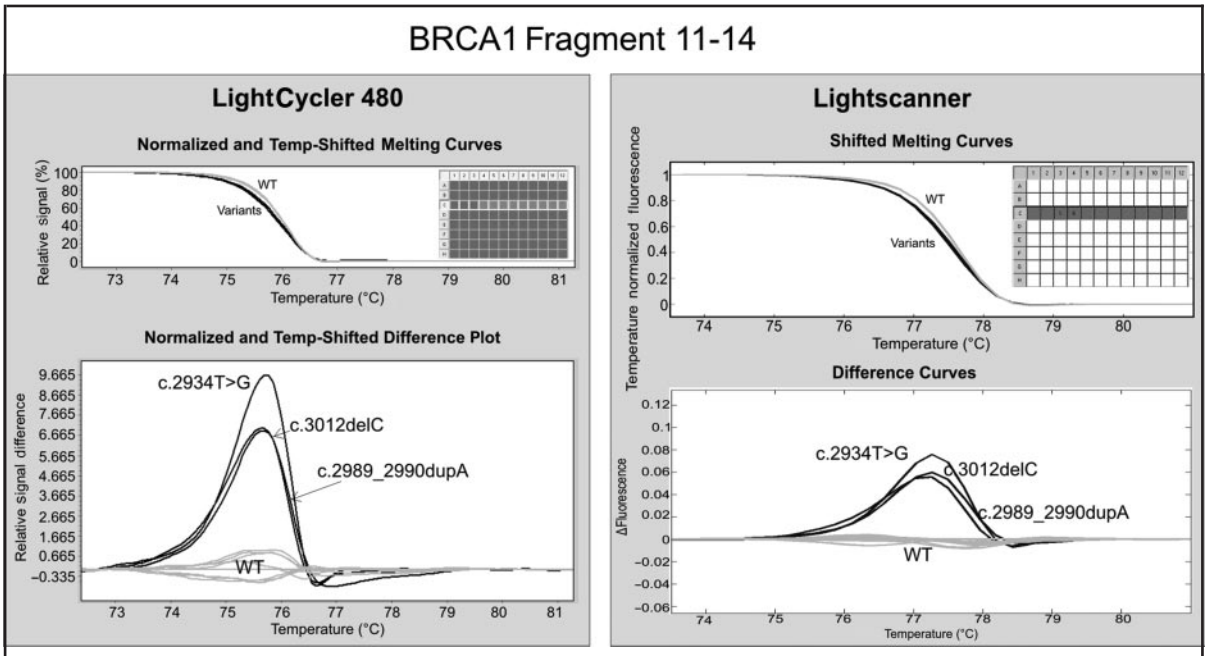

Fig. 3. Different sequence variants classified together by high-resolution melting.

Melting curves and difference plots for fragment *BRCA1* 11-14 on the Lightscanner and the LightCycler 480. A common polymorphism (*BRCA1* c.2934T>G) is grouped together with 2 distinct pathogenic mutations (*BRCA1* c.3012delG and *BRCA1* c.2989_2990dupA). These panels illustrate that every variation in the melting curves needs to be sequenced.

mozygous SNPs detected in the present study were indeed class 1 (C/T or G/A) and class 2 (C/A or G/T). The problem for the class 3 or 4 SNPs might be overcome by mixing samples with wild-type fragments. This could also be useful when high-resolution melting analysis is applied for mutational analysis of genes associated with recessive diseases or males with X-linked diseases.

From our observations, it became clear that the software is not always able to discriminate between distinct variants within the same amplicon. As an example, we found that the 2 SNPs *BRCA1* c.3113A>G and *BRCA1* c.3119 G>A were classified in the same group by the software (data not shown). This can be explained by the short distance and the small Tm variation between these 2 aberrations. In Fig. 3, however, we show a common polymorphism (*BRCA1* c.2934 T>G) grouped together with 2 distinct pathogenic mutations (*BRCA1* c.3012delG and *BRCA1* c.2989_2990dupA). This example illustrates that although high-resolution melting analysis is a very useful prescreening technique, all detected aberrations still need to be sequenced in a diagnostic setting. Recent advances in the use of unlabeled oligonucleotides will substantially reduce the sequencing work, as these approaches allow discrimination between different genetic variants within the same amplicon *(16)*. An alternative way for genotyping specific polymorphisms or distinct sequence aberrations was recently proposed by Dobrowolski et al. *(17)*. They designed a multiplex, short amplicon (146–266 bp) assay with primers flanking the aberrations and described the use of melt controls.

High-resolution melting is a mutation scanning technique suitable for the detection of point mutations. However, like other PCR-based techniques, large exon (or multi-exon) deletions will remain undetected. In some populations, large intragenic *BRCA1/2* deletions represent an important fraction of the mutation spectrum due to founder effects. For a complete mutation detection strategy, additional techniques such as multiplex ligation-dependent probe amplification (MLPA) are required.

Our experiences with high-resolution melting for the *BRCA1/2* genes allowed us to readily implement the technology for screening other large genes. However, preliminary data showed that pooling samples extracted in different laboratories produced variations in melting curves. We hypothesize that the differences in DNA-extraction methods may have influenced the results. The composition of the DNA solution buffer

might play an important role. The same findings were reported in a recent study of Seipp et al. *(18)*—amplicon Tm differences up to 0.39 °C were found when different DNA extraction methods were used.

In summary, we present a fast and reliable mutation detection strategy by high-resolution melting analysis on 2 different instruments. By introducing this method, our reporting time for the *BRCA* genes can be reduced considerably (one third compared to direct sequencing and DGGE). In our setup, the hands-on postmelt analysis of 11 patients requires only 3 h (approximately 13 min per 96-well plate) followed by sequencing of the detected aberrations. Owing to the relatively low cost of the consumables (LCGreen Plus; no need of fluorescence-labeled primers or special polymers) and the lower workload compared with other mutation scanning techniques, this is a very cost-efficient technology. The 2 high-resolution melting instruments evaluated were able to detect all known sequence variants. As suggested by Herrmann et al. *(10)*, we also found that the Lightscanner, specifically designed for high-resolution melting, displayed slightly better scanning specificity than the LightCycler 480, an instrument that can also be used for real-time Q-PCR. Further reduction of the sequencing burden can be obtained using unlabeled probes for the detection of frequent SNPs in both genes. We conclude that high-resolution melting is a rapid, cost-efficient, sensitive methodology simple enough to be readily implemented in a diagnostic laboratory.

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