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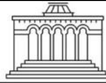

• *Փորձարարական և տեսական հոդվածներ* •*Экспериментальные и теоретические статьи* • *Experimental and theoretical articles* •

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HIGH-RESOLUTION MELT CURVE ANALYSIS AS A HIGH-THROUGHPUT SCREENING METHOD OF LARGE GENES

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High-resolution melt curve analysis (HRM) has been recently introduced as a promising technique for genotyping and mutation scanning in diagnostics. To screen the full coding regions and splice junction sites of BRCA1 and BRCA2 genes, amplification and HRM of 90 PCR amplicons were performed on the LightScanner-32™ (BioFire Diagnostics Inc.) instrument. Sensitivity of the method was evaluated by analyzing 82 clinically damaging mutations distributed in different amplicons and specificity by blind screening of 15 patients for BRCA1 and BRCA2. All known heterozygous variants were detected on the LightScanner-32 by analysis on normal sensitivity setting. 7 DNA-sequence variants had been detected among 15 patients, which all were confirmed by Sanger sequencing. Therefore, HRM is a cost-efficient, sensitive method suitable for high-throughput mutation screening for diagnostic purposes, particularly for large genes such as BRCA1 and BRCA2.

High-resolution melt curve analysis – BRCA1&2

Բարձր թույլատրելիության հալման կորի վերլուծությունը (high-resolution melt curve analysis; HRM) վերջերս է ներկայացվել որպես խոստումնալից մեթոդ ախտորոշման բնագավառում գենոտիպավորման և մուտացիաների սկանավորման նպատակով: BRCA1 և BRCA2 գեների ամբողջ կոդավորող և սպլայս-հատվածների սկրինինգի նպատակով 90 PCR ամպլիկոնների ամպլիֆիկացիան և HRM վերլուծությունը իրականացվել է LightScanner-32™ (BioFire Diagnostics Inc.) սարքի միջոցով: Մեթոդի զգայնությունը գնահատվել է, վերլուծելով կլինիկորեն 82 մուտացիաների բացահայտումը, որոնք տարածված են տարբեր ամպլիկոններում, իսկ սպեցիֆիկությունը՝ 15 հիվանդների BRCA1 և BRCA2 գեների կույր սկրինինգի միջոցով: Բոլոր հայտնի հետերոզիգոտ տարբերակները բացահայտվեցին սարքի նորմալ զգայնության կարգավորման դեպքում: 7 ԴՆԹ-հաջորդականության փոփոխություններ հայտնաբերվեցին 15 հիվանդների մոտ, որոնք բոլորն էլ հաստատվեցին Sanger-սեքվենավորման եղանակով: Այսպիսով, HRM ծեռնտու, զգայուն մեթոդ է, որը մատչելի է ախտորոշիչ նպատակներով մուտացիաների լայնածավալ սկրինինգի համար, մասնավորապես մեծ գեների պարագայում, ինչպիսին են BRCA1 և BRCA2:

Բարձր զգայնության հալման կորի վերլուծություն – BRCA1&2

Анализ кривой плавления с высокой разрешающей способностью (HRM) был введен недавно как метод генотипирования и скрининга мутаций в диагностических целях. Для сканирования полных кодирующих и сплайсинговых областей генов BRCA1 и BRCA2 были проведены амплификация и последующий HRM анализ 90 PCR ампликонов, с применением системы LightScanner. Анализ 82 клинически патогенных мутаций в различных ампликонах позволил оценить чувствительность метода, а специфичность метода была оценена слепым скринингом генов BRCA1 и BRCA2 у 15 пациентов. Все известные гетерозиготные варианты были выявлены программой нормальной чувствительности прибора. Среди 15 пациентов были обнаружены 7 вариантов последовательностей ДНК, которые были подтверждены методом Sanger-секвенирования.

Следовательно, HRM – эффективный, чувствительный метод, подходящий для высокопроизводительного скрининга мутаций в диагностических целях, особенно при исследовании генов больших размеров, таких как BRCA1 и BRCA2.

Анализ кривой плавления с высокой разрешающей способностью – BRCA1&2

BRCA1 (MIM 113705) and BRCA2 (MIM 600185) both encode relatively large proteins, and the majority of predisposing mutations in these genes are protein-truncating mutations that cause substantial loss of function predisposing to breast and/or ovarian cancer. BRCA1 is a large gene consisting of 24 exons encoding 1863-amino acid protein [5]. BRCA2 is a larger gene consisting of 11,385 protein-coding nucleotides in 27 exons. To date, more than 1000 and 500 deleterious mutations (including frameshift, missense, nonsense, and splice site) have been found in BRCA1 and BRCA2, respectively [1]. The majority of laboratory approaches available for predisposition diagnostic testing fall into one of five categories: (i) conformational analyses; (ii) mismatch/heteroduplex analyses; (iii) protein truncation assays; (iv) tests for rearrangements; and (v) (sequencing). In addition to detecting the presence of a genetic variant in a patient, there are two additional requirements on the testing process: (vi) sample tracking and (vii) genetic variant classification.

The method of HRM [3, 4] rests on three biophysical principles. First, certain dyes like LCGreen® Plus bind to double-strand DNA, fluoresce under ultraviolet light when bound, are compatible with PCR at saturating concentrations, and increase tremendously the sensitivity and specificity of DNA melting analysis. This makes high-resolution fluorescence versus temperature, or melting curve, analysis of PCR products by optical methods. Second, if a particular amplicon is polymorphic, PCR of that amplicon from a heterozygous sample followed by denaturation and reannealing results in four DNA duplexes: two homoduplexes of opposite genotype and two mismatched heteroduplexes. Each of these duplexes has a characteristic melting curve, and the sum of all of the melting curves of the PCR products present in a single sample can be observed by a HRM analysis. Third, because mismatched heteroduplexes have a lower Tm than either homoduplex, a heterozygote in the sample (and resulting mismatch duplex) shifts the melting curve profile. This shift can be detected reliably by a suitable sensitive HRM instrument.

In this study, high-resolution melt analysis for BRCA1 and BRCA2 was evaluated on the LightScanner-32 (BioFire Diagnostics Inc.) instrument. Sensitivity and the specificity of the method were determined by the analysis of 82 known deleterious controls and a blind screening of 35 patients (1432 PCR reactions).

Materials and methods. *DNA samples and study design.* The DNA samples tested for BRCA1&2 included 82 variants and 10 wild-type controls, which were all verified by direct sequencing using the Big Dye Terminator method (LifeTechnologies). 51 positive BRCA1 samples scattered over 36 of 40 amplicons were available, including 8 1-3 bp deletions, 5 1-bp insertions, 23 transitions, and 15 transversions. 31 positive BRCA2 samples scattered over 31 of 50 amplicons were available, including 11 1-4 bp deletions, 3 1-bp insertions, 12 transitions and 5 transversions. Positive samples for 23 amplicons were missing.

To evaluate the specificity of the method, 15 patients were chosen and blindly screened for BRCA1 and BRCA2 in parallel with direct sequencing of all amplicons using the Big Dye Terminator method.

Primers. Two panels of 80 and 100 primer pairs were evaluated for HRM analysis of full coding region and splice junction sites of BRCA1 and BRCA2, respectively. The primers were newly designed or derived from current primer sets in use for direct sequencing of both genes. The design of most new primers was performed using the Oligo7 Primer Analysis software (Oligo7).

All primer sets contain M13 forward and reverse sequence tails to allow direct sequencing analysis, and were synthesized by Biolegio Inc.

PCR, melting acquisition and analysis. Both PCR amplification and HRM analysis were performed on the 32-well LightScanner instrument allowing sequential performance of two procedures. 10 µL PCR mixtures, including 4 µL of LCGreen master mix with Taq polymerase and dNTPs, 3.4 µL deionized water, 3 pmol/L of both forward and reverse primers, and 20 ng DNA were transferred to individual capillaries suitable for LightScanner-32 and were covered with a mineral oil. The amplicon length ranged between 190 and 530 bp. All 90 amplicons were amplified using a universal PCR-HRM program. The temperature cycling protocol consisted of an initial denaturation step at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 20 sec, annealing at ranging from 50-66°C for 30 sec (ramp rate 2.5°C/sec), and extension at 72°C for 40 sec. Finally, reactions were elongated for 5 min at 72°C and heteroduplexes were generated by adding a step at 951C for 1min and cooling the reactions to 25°C. Ramp speed of the instrument was set at 2.5°C/sec. Melting curve analysis was performed on the LightScanner-32 software with the ‘‘Scanning’’ mode used for standard mutation scanning, employing a 3-step analysis by: 1) selecting negative controls, 2) normalization of the melting curves, and 3) curve shift to normalize temperatures and group melt profiles of similar shape.

Characterization of the genetic variants by Sanger sequencing. PCR products displaying a melting curve that differs from the reference group were re-arrayed into 96-well plates and treated with exonuclease I and shrimp alkaline phosphatase to remove excess primers and nucleotide triphosphates (exo-SAP treatment). Dye-terminator sequencing reactions (BigDye Terminator, version3.1, LifeTechnologies) were inoculated with the exo-SAP-treated PCR products, thermocycled, and then purified with Montage SEQ96 sequencing reaction clean up kits (Millipore). Sequencing reaction products were then run on a 96-capillary Spectrumedix Sequencer (Transgenomics) in accordance with the manufacturer’s recommendations. Process and data management of the mutation screening were carried out as described previously [(2, 6, 7].

All samples found to carry a rare sequence variant were re-amplified from genomic DNA for confirmation of the presence of the variant. DNA samples from all 15 breast cancer patients were screened for genetic alterations in the entire coding sequence plus splice junction boundaries of BRCA1 and BRCA2.

Results and Discussion. Germline BRCA1 and BRCA2 mutations are dominant, and rarely two mutations are found in cis position. Therefore HRM analysis is ideal for the screening of heterozygous sequence variants of BRCA1 and BRCA2. 51 known BRCA1 heterozygous sequence variants spread over 36 of 40 amplicons and 31 known BRCA2 sequence variants spread over 31 of 50 amplicons were analyzed with HRM on the LightScanner-32 instrument. The melting curves of the positive controls were compared with those of 10 healthy individuals. Positive samples for 23 amplicons were missing, and the quality of amplicons were evaluated by HRM analysis of by analyzing 10 wild-type samples. Totally, 737 amplicons were screened. All 51 and 31 known heterozygous BRCA1 and BRCA2 sequence variants, respectively, were detected by HRM analysis with the normal (default) sensitivity. In addition, 6 single nucleotide polymorphisms (SNPs) were detected in 10 healthy control individuals and confirmed by Sanger sequencing. Thus, all types of single nucleotide variations, small deletions and insertions were easily detected by HRM analysis on the LightScanner-32 instrument.

To determine the specificity of HRM analysis, we performed a blind screening of 15 patients for BRCA1 and BRCA2. All 90 amplicons of 15 patients were screened by HRM analysis at the default sensitivity, and in parallel were sequenced. All sequence variants were detected on the LightScanner-32, confirming the 100% sensitivity. Compared to 100% of testing sensitivity after the detection of all known sequence variant on the instrument, specificity of 98.96% was detected because of 24 false positive samples confirmed by Sanger sequencing among all 1350 amplicons.

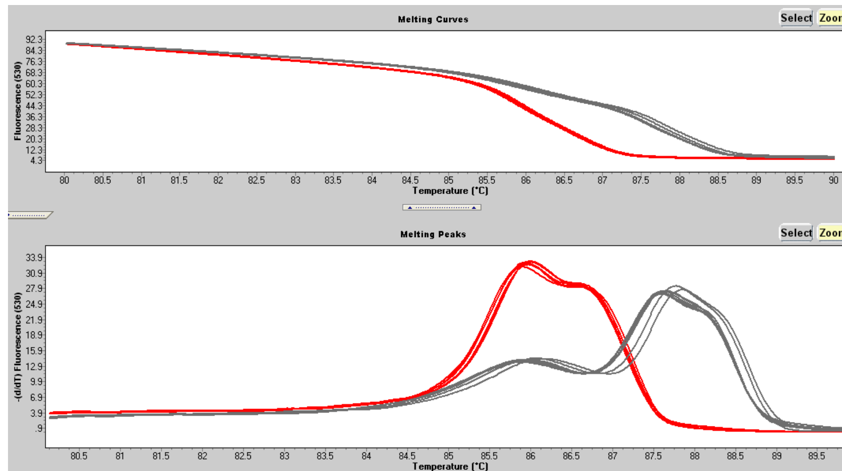

Fig. 1. Selection of negative controls.

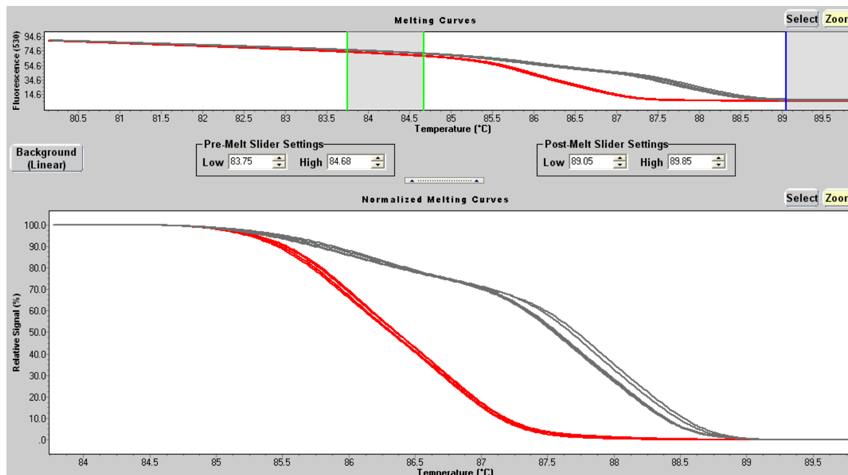

Fig. 2. Grouping of similarly melted samples by temperature shift

The aim of this study was to adapt and evaluate BRCA1 and BRCA2 mutation detection by the HRM analysis on the LightScanner-32™ (BioFire Diagnostics Inc.) specially designed for high-resolution melting analysis. Extensive mutation screening of high-risk breast cancer primarily targeting early-onset cases could be easily undertaken if there is a high-throughput technique allowing rapid and relatively inexpensive detection of mutations.

For this purpose, 90 PCR amplicons were designed covering the full coding and splice regions of BRCA1 and BRCA2. Both high-throughput PCR reactions and the following high-resolution melting analysis were performed on the same instrument.

The sensitivity and specificity of high-resolution melting was evaluated by the analysis of 1432 PCR amplicons. We detected all known sequence variants on the LightScanner-32 (normal sensitivity setting). Previously, specificity and sensitivity of HRM for mutation scanning of snigle-base substitutions were evaluated using an assay specifically developed to assess the effect of PCR product size, GC content, and the

nature and position of the base change within the amplicon [4]. The authors reported 100% sensitivity for SNPs in fragments of less than 300 bp and over 95% sensitivity in fragments with lenght between 400 and 1000 bp. The false-negative error rate tended to increase with fragment length and for the identification of A:A ot T:T mismatches.

Technical assessment of the protocol by the UK National Genetics Reference Laboratory also reported over 98% sensitivity for a mix of small insertion-deletion mutations and SNPs in a variety of sequence contexts [8]. Therefore, for high values of sensitivity and specificity, PCR conditions have to be optimized to have reproducible results for the same amplicons.

The data showed that the melting analysis could not discriminate close sequence variants within the same amplicon with small Tm difference, and classified hem in the same group (data not shown). Therefore, all detected aberrations should be sequenced, since there is a strong probability that this will become a popular mutation-screening diagnostic method.

HRM offers several obvious advantages as compared to traditional mutation scanning methods. First it is a rapid and secure method minimizing any risk of sample contamination. Second, HRM is easy to implement as it does not require post-PCR manipulation other than closed-tube melting analysis. Third, it is extremely easy to automate with few steps. Fourth, the melt curve analysis is nondestructive; individual samples can be reanalyzed and also be sequenced. Fifth, the protocol is relatively inexpensive requiring only PCR reagents plus small amount of fluorescent dye. Finally, HRM is suitable for high-throughput mutation screening enabling the simultaneous acquisition of fluorescent melting signals in less than five minutes.

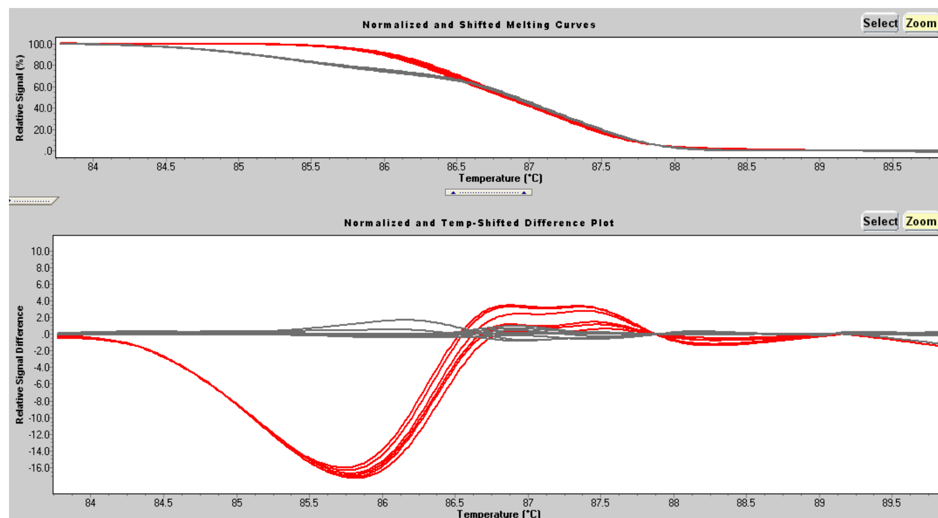

Fig. 3. Melting curves and difference plots of same sequence variants for exon 2 of BRCA1

HRM analysis of BRCA1 and BRCA2 is a fast, cost-effective, and reliable mutation detection strategy with lower workload for diagnostic purposes. LightScanner-32 detected all known sequence variants, giving a sensitivity value of 100%. Detection of common polymorphisms with unlabeled probes will substantially reduce the load and price of further sequencing reactions.

HRM analysis of BRCA1 and BRCA2 could be undertaken in low- or middle-income countries as a high-throughput screening method of BRCA1 and BRCA2 and

could be paired with proper genetic counselling to help affected women and their at-risk relatives understand the genetic risk factors of breast cancer. Hence, personal risk information may help in taking preventive measures and also motivate a high-risk woman to adopt breast screening that may promote early detection and improve chances of surviving for these patients.

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