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A Novel HRM Strategy for Detection of BRCA Mutations on Mexican Population

Lugo FI, Diaz NIO, Barbosa LC and Aguirre G*

[1]*Laboratorio de Genética y Biología Molecular, D-SU Biotek S.A. de C.V, México*

***Corresponding author:** Gisela Aguirre, Laboratorio de Genética y Biología Molecular, D-SU Biotek, S.A. de C.V. Paseo de los Héroes 10231 Int.301 Zona Urbana Rio, 22010, Tijuana, Baja California, México, Tel: +52(155) 1227 2200; E-mail: gisi@dsubiotek.com

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Introduction

Breast cancer (BC) is the most prevalent malignancy in women in both developed and less-developed countries [1-9]. In 2012, 1.67 million new cases were diagnosed worldwide, representing 25% of all cancers [10]. BC is the major cause of mortality in both developed (198,000 deaths, 15.4% of total; ranking second after lung cancer) and less-developed (324,000 deaths, 14.3% of total; ranking first) countries. About one in 12 women in the West develop BC at some point in their life [11].

In 2014, the American Cancer Society estimated that about 235,030 new cases of BC would be diagnosed [12] and approximately 10% of these cases would be hereditary [13]. There are approximately 35,000 living BC patients with a BRCA mutation but only 30% of them have been identified. In the United States, there are around 220,000 BRCA mutation carriers and fewer than 10% have been identified [14].

Once detected, BC treatment involves chemotherapy and in some cases surgery. Both toxic and trauma treatments may be avoided through effective prevention and surveillance strategies. Genetic testing has an important role in hereditary risk assessment for BC. As a result of changes in patent laws and gene sequencing technologies improvements, there has been a rapid expansion of genetic testing. To date, there are several genetic tests currently on the market [15] that screen from known point mutations and small deletions, to large chromosomal rearrangements.

BRCA1 and BRCA2 are the most important genes predisposing to inherited breast and ovarian cancers [16]. Germ line mutations in these two highly penetrant genes can increase the lifetime risk of developing BC by as much as 80 %, and these mutations are also associated with an earlier onset of the disease. Moreover, different cancers (i.e. prostate, pancreatic and gastric cancers) are more common in BRCA1 and BRCA2 mutation carriers than in general population [17]. Ethnicity is also a risk factor since prevalence of BRCA mutations varies significantly across racial and ethnic groups [18,19].This is relevant since the data and conclusions derived from a specific population might not be true for a different population, and this will help to get the maximum benefits from all the strategies and diagnostic tools available. Furthermore, in December 2014, a poly(ADP-ribose) polymerase (PARP) inhibitor was granted expedited approved by the United States Food and Drug Administration for use in advanced ovarian cancer patients with germ line BRCA1/2 mutations who have received three or more prior lines of chemotherapy. The mentioned compound named Olaparib, has also shown to reduce the growth rate of cancer in BRCA mutation positive patients in preclinical phases [20-21]. Therefore, there is increasing evidence that emphasizes the importance to identify BRCA mutations. Additionally, early identification of BRCA mutations among women affected by BC is crucial to enable patient stratification and to guide clinicians in deciding the most appropriate therapeutic strategy and follow-up program. Accurate genetic counseling can identify at-risk healthy members of affected families, who can be involved in appropriate surveillance programs.

Direct Sanger sequencing continues to be the routine procedure for the molecular analysis of the BRCA genes [24,25]. However, given the large size of both BRCA1 and BRCA2 and the consequent cost of their direct sequencing, large-scale mutation scanning strategies such as denaturing high- performance liquid chromatography (dHPLC) and high-resolution melting, are the major strategies used as pre-sequencing methods [24,25].

However some of these alternatives for genetic screening are expensive, they need specifically trained professionals and the overall process can increase anxiety in patients or generating fear around the choice of knowing their own actual status and thus delaying the start of treatment.

High resolution melting curve (HRM) analysis detects nucleotide variations within a targeted gene region [26]. The basis of HRM relies on the melting behavior of double-stranded PCR product. This method whereby amplicons have potentially distinguishable melting profiles, detects differences in the sequences even by a single nucleotide. The melting profiles can be monitored using saturating dyes, which show fluorescence when intercalated within double-stranded PCR products. HRM analysis is advantageous as a closed-tube method, involving no post- PCR manipulations. Several studies have been conducted to scan BRCA mutations by the HRM analysis approach [27-32]. This method starts with PCR amplification of the region of interest in the presence of a fluorescent dsDNA-binding dye. When the dsDNA dissociates (or melts) into single strands, the dye is released, causing a change in fluorescence. Then, in HRM experiments, after PCR, the product is gradually melted and the number of fluorescent data points generated is registered by using high precision instrumentation that measures changes every °C. Fluorescence measurements are plotted to create a melt curve (or profile). Melting curves are generated by monitoring the fluorescence of a saturating dye. Identification of heterozygotes is made by a change in melting curve shape, whereas homozygous Single Nucleotide Polymorphism (SNP) genotypes are distinguished by a change in melting temperature (Tm).

Here, we report the use of a new HRM based analysis approach to perform allele specific expression measurements in BRCA1 and BRCA2 genes in Mexican population. We analyzed 185delAG, 330A>G, 4446C>R1443X, IVS20+12 and 5382InsC mutations for BRCA1; 3492InsT 6174delT and 7105T>CF2293L mutations for BRCA2. These particular mutations were selected based on

bibliographic research, focusing on the most probable mutations on Mexican population. We confirmed that this methodology is able to detect in less than 2 hours, not only the expected mutation, but different sequences, some of which have not been described previously.

Material and Methods

Reagents

The QI Amp DNA Investigator kit (catalog number: 56504) was purchased from Qiagen (Venlo, NL). Oligonucleotide primers were acquired from T4; Oligo Company (Irapuato, Mexico). Agarose molecular biology grade was obtained from SIGMA (MO, USA). Gel red was purchased from Biotek (VT, USA). Luminaries color HRM qPCR master mix (catalog number: K1031BID) was purchased from Thermo Scientific (MD, USA).

Samples and Nucleic Acid Isolation

Recruitment of volunteer participants and sample obtaining

Recruitment of volunteers was made by self-referral. Study information/leaflets were made available through hospitals. Volunteers received structured, nondirective pretest genetic counseling for informed decision-making. Inclusion criteria were: female gender, age greater than 18 years, Mexican ethnicity (self-reported history, four Mexican grandparents); and diagnostic of BC, or possessed BC familiar risk. 36 peripheral blood samples were obtained from volunteer screening.

PCR and HRM Analysis

DNA was isolated using DNA investigator kit following manufacturer instructions. To ensure good discrimination between wild type and mutant, effective primer design is one of the most important parameters in HRM assay. The proprietary sequences of the primers, designed to flank the coding regions of BRCA1/BRCA2 genes, are actually under a copyright process. The general information for these primers is listed in Table 1. GenBank sequences NM_007294.3 and NM_000059.3 were used as reference for BRCA1 and BRCA2 genes respectively.

Gene	Mutation	PCR Product Size, bp
BRCA1	185delAG	91
	330A>G	88
	4446C>R1443X	108
	IVS20+12	198
	5382InsC	118
BRCA2	3492InsT	114
	6174delT	117
	7105T>C F2293L	92

Table 1: List of oligonucleotides and mutations selected for BRCA1 and BRCA2 mutations screening.

Endpoint PCR was made on a MaxyGene Thermal Cycler from Thermo Scientific and PCR conditions were as follows: initial denaturation at 95°C for 5 min and 30 cycles of denaturation at 95°C for 5 sec, annealing at 55°C for 15 sec and extension at 72°C for 15 sec. A final extension step of 72°C for 1 min was added.

The qPCR and HRM were performed in a single run on a Light cycler 96 (Roche) using the Luminaries color HRM qPCR master mix kit which uses Eva Green as fluorophore. Samples were run in duplicate using 50ng of genomic DNA, 0.5µM of forward and reverse primers. Agarose gels were run for each primer set to ensure that the correct size amplicon was produced and that there were no non-specific amplicons or primer dimers formed.

Real time PCR conditions were as follows: 95°C for 10 minutes as initial denaturation, and 45 cycles of 95°C for 10 sec, 55°C for 15 sec and 72°C for 15 sec for extension. The HRM analysis was performed after qPCR reaction as follows: 95°C for 1 minute for denaturation, 40°C for 1 min for renaturation, and a continuous ramp from 60°C to 95°C with 25 readings per °C. The reaction was cooled at 40°C for 10 sec.

Results

36 blood samples were isolated by the QI Amp DNA Investigator kit, and analyzed for HRM using the oligonucleotides described in Table 1. These primers were tested on endpoint PCR to verify their specificity and discard possible unwanted amplicons that can interfere with further HRM analysis. In Figure 1 we can see the amplification products of our set of primers. As it is shown, only one expected size amplicon per primers set was produced and no other additional PCR products. After this result we proceed to make the HRM analysis by real time PCR.

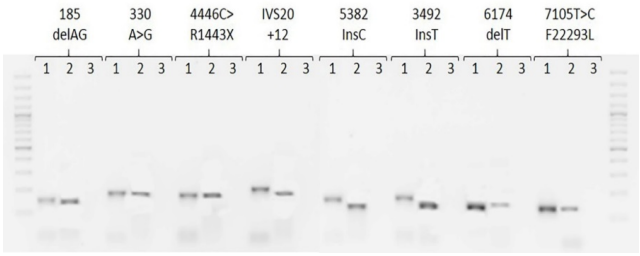

Figure 1: Endpoint PCR products for BRCA1 and BRCA2 mutations using positive controls for each mutation. Is clear to see that we have only one amplification product for each one of the mutations. Positive controls are synthetic oligonucleotides with the sequence for each mutation. Lanes: 1, Positive control for wild type (WT) sequence; 2, Positive control for mutated sequence; 3, Negative control where no template was added.

After HRM analysis we detected two samples positive for mutations across the regions where 185delAG and 3492InsT were flanked. We were able to detect variations in these two sequences, which confirm the effectiveness of the method (Figure 2) in finding mutations. Previously, our group reported the 185delAG mutation as the second most prevalent BRCA1 mutation in Mexican population. Whereas mutation 3492InsT is also significantly present in Mexicans [33].

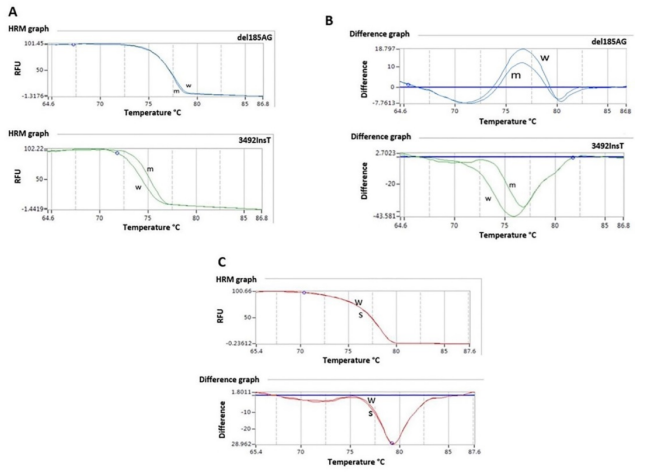

Figure 2: HRM analysis for one sample of BRCA1 (del185AG) and one for (BRCA2 3492Inst), panel A shows the HRM plot for BRCA1 and BRC2, and panel B shows the difference plot of the same positive samples were we can see the behavior of the amplification product melting and the differences between the samples and synthetic sequences of the wild type phenotype. Panels C shows both the HRM graph and the difference graph between the sample of one healthy volunteer and synthetic control for one mutation in the BRCA1 gene (BRCA1 330AG) showing no difference between them, this to show the specificity of our method.

To validate our results, we sequenced the regions where mutations were identified by HRM. Sequencing results are summarized on Table 2. In sample A we did not detect specifically the 185delAG mutation, however we were able to detect 8 different sequences in this region. The founded differences were mostly single nucleotide mutations (insertions and changes of one base) excepting the insertion of three bases in position 219.

Sample A BRCA1 polymorphisms sequencing results			
Type of mutation	**Nucleotide**	**Position**	**Mutation**
Insertion	A	185	185insA
Insertion	T	204	204insT
Change	C > A	211	211C>A
Change	A > C	212	212A>C
Change	G > A	214	214G>A
Change	T > A	216	216T>A
Insertion	GTA	219	219insGTA
Change	A > T	220	226A>T
Sample B BRCA2 polymorphisms sequencing results			
Change	A > T	3419	3419A>T
Deletion	T	3421	3421delT
Change	A > C	3487	3487A>C

Table 2: Sequencing results on one region of sample A encompassing the exon 11 and sample B which corresponds to BRCA1 and BRCA2 respectively. Here we can detail the sequence variations for BRCA1 and BRCA2, where we found different single nucleotide mutations not previously reported. Only the Sample A 185insA has been reported in the BIC database.

Similar results were found in sample B, where we did not specifically detect the expected InsT mutation, but presented 3 different mutations in this BRCA2 gen region. The founded differences in sample B, corresponded to single nucleotide mutations: two changes and one deletion (Table 2).

DISCUSSION

Identification of founder and recurrent mutations is a fundamental step towards the improvement of genetic counseling. In the case of BC, this improvement relies on the identification of related mutations present in specific ethnic groups, which eventually will be the basis for the development of rapid and less expensive test for determined human population.

In order to detect potential mutations and, at the same time, avoid sequencing the entire BRCA genes, several alternate methods have been suggested for screening. The purpose of our study was to evaluate the efficacy of the HRM method in screening BRCA1/2 mutations in a sample of Mexican population. High Resolution DNA Melting Analysis was first described in 2003 [33] and it has been used to screen many different mutations relevant in cancer, specifically breast cancer related mutations on BRCA genes [33-36].

To date there are few studies on Mexican population regarding the specific BRCA mutations. Recently we performed a meta-analysis to look for some of the most probable mutations affecting this particular ethnic group [34]. These results together with a previous bibliographic research focused exclusively on Mexican population, lead us to select the mutations named in this work. With HRM method we are able to detect different sequence variations in BRCA1 and BRCA2 genes. Surprisingly, we did not find the classic reported mutations we were looking for BRCA1 (185delAG) and BRCA2 (3492InsT). However, it is important to note that this type of methodology is known for its potential in discovering new mutations, since HRM is not sequence specific and allows the detection of any change in the melting curve profile as a result of the presence of any sequence alteration (known or unknown)[35,36].

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