

A novel method to detect the Mexican founder mutation *BRCA1* ex9-12del associated with breast and ovarian cancer using quantitative polymerase chain reaction and TaqMan[®] probes

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Abstract. In 2015, according to the National Institute of Statistics and Geography (INEGI), malignant breast tumors were the first cause of cancer fatality in women (6,273 fatalities) in Mexico, whereas 2,793 fatalities in women were due to ovarian cancer. A total of 5-10% of breast cancer and 10-15% of ovarian cancer cases are caused by a hereditary breast-ovarian cancer syndrome, with mutations predominantly identified in the *BRCA1* and *BRCA2* genes. Recently, the Mexican founder mutation *BRCA1* ex9-12del was identified (deletion of exons 9-12 with recombination between introns 8-12). This is the most frequently reported mutation in hereditary breast/ovarian cancer in Mexico. Current detection methods include end-point polymerase chain reaction (PCR) and Multiplex Ligation-dependent Probe Amplification (MLPA). In the present study a cheap, sensitive and fast detection method was developed based on quantitative PCR and two TaqMan[®] probes, one to detect the deletion (recombination region between introns 8 and 12), and the other one a region from exon 11. With this assay, 90 samples were able to be analyzed in 2 h using 2.5 ng of DNA/reaction at a cost of ~2-3 USD. This method is capable of detecting positive samples for DNA

deletion and excluding negative ones. Therefore, the method proposed may be a useful high-throughput diagnostic option that could be useful in future association or prevalence studies that use large populations.

Introduction

Current diagnostic tools for detection of breast cancer have contributed to reduce its mortality. However, in 2015, the National Institute of Statistics and Geography (INEGI) in Mexico reported that breast cancer was the first cause of death among women between 35 and 44 years old (1). Additionally, ovarian cancer was within the top ten causes of cancer death in Mexican women (2).

Despite recent advances in science and new approaches for the detection of ovarian cancer through liquid biopsy, such as salivary samples, (3) in Mexico there are still no effective methods to reduce mortality due to ovarian cancer. Transvaginal ultrasound is the most commonly used technique but it might not always detect early stage abnormalities. In addition, there is no self-examination method as there is for breast cancer (4). In most cases, ovarian cancer is diagnosed when the tumor is already in an advanced stage.

Both breast and ovarian cancer share several risk factors, such as advanced age, nulliparity or having a full term pregnancy after 30-35 years old, family history of breast or ovarian cancer, mutations in *BRCA1* and *BRCA2* genes, early menarche, not having breastfed, late menopause and obesity (5-10). Among 5-10% of breast cancer and 10-15% of ovarian cancer cases are due to a hereditary mutation (11). Hereditary Breast and Ovarian Cancer syndrome (HBOC) is characterized by a high risk of developing breast or ovarian cancer (12). Most of the mutations found in HBOC are localized in the tumor suppressor genes *BRCA1* and *BRCA2*, which participate in DNA repair. Carriers of a *BRCA1* mutation have a risk of

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65-85% and 39-44% of developing breast and ovarian cancer, respectively, whereas having a *BRCA2* mutation confers a risk of 45-80% of developing breast cancer and 11-27% for ovarian cancer (11). Several mutations have been identified in specific populations, for example, in Ashkenazi Jews, the *BRCA1* mutations 185delAG and 8382insC, and the *BRCA2* mutation 6174delT are found within 2-3% of the population (13). These mutations have been identified as founder mutations, because they were originated in a common ancestor and then inherited through generations (14). In 2007, the Mexican founder mutation *BRCA1* ex9-12del, a deletion of exons 9-12 of around 14.7 kb, was reported for the first time (15). Approximately, 42% of the *BRCA1* gene is composed by *Alu* repetitive sequences, which can lead to errors during homologous recombination (16). Consequently, the *BRCA1* ex9-12del mutation arises due to a recombination event between an *AluSp* element in intron 8 and an *AluSx* element in intron 12, with the subsequent loss of exons 9-12 (15).

BRCA1 ex9-12del mutation was first documented in a sample of women with Hispanic ancestry and family history of breast and ovarian cancer. The deletion was found in four non-related families, all of them with Mexican ancestry (15). The same research group performed another study in Hispanics, in which individuals with personal or family cancer history were shown to have *BRCA* mutations; 21 of 189 were specifically large rearrangement (LR) mutations, in which 13 were carriers of the *BRCA1* ex9-12del mutation (17). In a different study involving Mexican women with breast or ovarian cancer, the *BRCA1* ex9-12del mutation was present in 35% of ovarian cancer cases associated with *BRCA* and in 29% of breast cancer cases associated with *BRCA* (18). In a later study where only women with breast cancer participated, the deletion was found in 24% of cases associated with *BRCA* mutations, in patients between 35 and 56 years old (19). A more recent report showed that the *BRCA1* ex9-12del mutation represented 42% of the cases with *BRCA* mutations among young Mexican women diagnosed with triple negative breast cancer (20). Regarding the origin of the *BRCA1* ex9-12del mutation, it has been suggested that it arose 1480 years ago (95% CI, 920-2260 years) in Puebla, Mexico (17). Studies performed in high breast cancer risk individuals from Spain, Colombia, Germany and Pakistan, have shown the absence of the mutation in those populations (21,22). Moreover, reports from Myriad Genetic Laboratories (Salt Lake City, UT) indicated that patients with HBOC of Latin American/Caribbean ancestry commonly carry large rearrangements (21.4% of all *BRCA* mutations). In these reports, the Mexican founder mutation *BRCA1* ex9-12del accounted for 37% of those LR (23).

Taken together, these data show the high frequency of the *BRCA1* ex9-12del mutation among young Mexican women with HBOC. Therefore, the detection of this deletion in relatives of the mutation carriers might encourage strategies for early detection and prevention of breast and ovarian cancer, such as recurrent clinical and imaging examinations, lifestyle modifications or prophylactic surgeries and/or chemoprevention use. Moreover, analyzing the presence of this deletion in Mexican patients suspicious of carrying a *BRCA* mutation will definitely be a cheaper option, before searching for mutations and LR in complete *BRCA* genes. Sequencing of both *BRCA* genes costs ~1,000-4,000 USD (24). Myriad Genetic

Laboratories currently uses quantitative multiplex endpoint polymerase chain reaction (PCR) to detect a set of common LR, including the *BRCA1* ex9-12del mutation, at a cost of 700 USD, under the name BRACAnalysis Large Rearrangement Test (BART) (23,25). Unfortunately, most Mexican patients do not have access to such expensive tests. Other techniques can be used to detect LR, for example Multiplex Ligation-dependent Probe Amplification (MLPA), Protein Truncation Test (PTT), long range PCR, Fluorescence *In Situ* Hybridization and end-point PCR (26). However, these techniques can be time consuming, difficult to interpret and expensive since different equipment and reagents are required. Most of the research papers that have screened patients for the *BRCA1* ex9-12del mutation have used end-point PCR (15,18,20), MLPA (19) and one included the Myriad diagnostic test (17).

The aim of the present study was to develop a new method to detect the Mexican founder mutation *BRCA1* ex9-12del by qPCR and TaqMan® probes. The method we propose is economic, specific, sensitive, fast, easy to interpret, and it can be used in both research and diagnostic scenarios.

Materials and methods

Study samples. A total of four DNA samples from previously known carriers of the *BRCA1* ex9-12del mutation were obtained, three from the Hospital Zambrano Hellion TecSalud and one from the Laboratorio Nacional Biobanco. Control samples were obtained from six female volunteers without a family history of cancer, who work at the Universidad de Monterrey. Three ml of peripheral blood were collected in a tube with EDTA. The study complied with the guidelines of the Declaration of Helsinki and was approved by the Research and Ethics Committee from the University of Monterrey (registration no. 01072017-CIE). Written informed consent was obtained from all subjects.

DNA extraction. The Wizard Genomic DNA Purification kit (Promega Corp., Madison, WI, USA) was used to isolate DNA from the six volunteers' blood samples. DNA extraction was performed according to manufacturer's instructions. Genomic DNA was quantified by UV absorbance using Nanodrop (Thermo Fisher Scientific, Inc., Wilmington, MA, USA). The DNA quality was evaluated with the A260/280 and A260/230 ratios. Samples were stored at -20°C.

Characterization of the recombination region between introns 8 and 12 of *BRCA1*. The key contribution of our work was the design of a TaqMan® probe which is complementary to the recombination region between introns 8 and 12 of *BRCA1*, for the detection of the *BRCA1* ex9-12del mutation. In order to achieve this, the first step was to identify the DNA sequence of the recombination site. Therefore, an end-point PCR assay was designed (Fig. 1), and then the PCR product was sequenced. The DNA of a sample from a *BRCA1* ex9-12del mutation carrier was amplified using the Go-taq flexi DNA polymerase kit (Promega Corp.). The PCR was carried out in a total volume of 15 µl containing 100 ng of DNA, 1X buffer, 1.5 nM MgCl₂, 0.2 mM dNTPs, 0.2 µM primer F, 0.2 µM primer R, 1.25 units of DNA polymerase and nuclease-free water. Initial denaturation was set at 94°C for 3 min, then 30 cycles of denaturation at 94°C

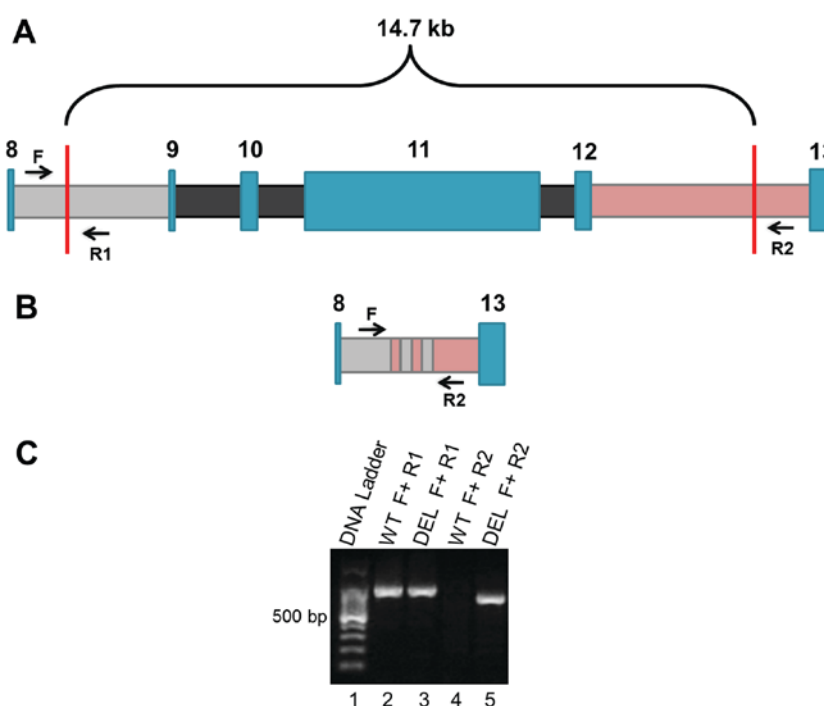


Figure 1. Location of the *BRCA1* ex9-12del mutation and design of the end-point PCR method for its detection. (A) Wild-type allele of the *BRCA1* gene showing exons (blue color) and introns (8 gray color; 9, 10 and 11 black color, 12 pink color). In the *BRCA1* ex9-12del mutation, the missing DNA includes exons 9 to 12 (14.7 kb) in one single copy of the gene (heterozygous), having the breakpoint somewhere in introns 8 and 12 (vertical red lines). Black arrows indicate the design and position of the primers used for the end-point PCR assay (F, R1 and R2). (B) Variant allele with the deletion of exons 9-12 and the recombination of introns 8 and 12 (pink/gray zone). Black arrows indicate position of primers F and R2. (C) Agarose gel electrophoresis after the end-point PCR of a sample carrying the mutation (DEL) and a wild-type sample (WT). Lane 1, DNA ladder. Lanes 2 and 3 represent the amplification of samples WT and DEL with primers F and R1, showing an amplified product corresponding to a region of the wild-type allele (~840 bp). Lanes 4 and 5 are the PCR products of a WT sample and a DEL sample respectively, with primers F and R2, amplifying the allele with deletion only (~630 bp). This PCR fragment can be seen only in samples DEL. Primers F and R2 in a wild-type allele would give an amplified PCR product >14.7 kb, an impossible task for our end-point PCR. PCR, polymerase chain reaction.

for 30 sec, annealing 40 sec at 60°C for primers F and R1 or at 55°C for primers F and R2, extension at 72°C for 1 min. A final extension was included at 72°C for 5 min. The reaction was performed in a Thermocycler Eppendorf Mastercycle EP gradient S Thermal (Eppendorf, Hamburg, Germany). The PCR products were analyzed by electrophoresis on a 1.5% agarose gel and stained with GelGreen™ (Biotium, Fremont, CA, USA). Primers were designed using the *BRCA1* DNA sequence with accession no. L78833.1 from the GenBank of the National Center of Biotechnology Information (NCBI; <https://www.ncbi.nlm.nih.gov/nuccore/L78833.1>).

Sequencing of the recombination region between introns 8 and 12 of *BRCA1*. The PCR product amplified with primers F and R2 of a sample from a *BRCA1* ex9-12del mutation was purified with QIAquick kit (Qiagen, Hilden, Germany) following manufacturer's instructions. The sample was sequenced in a Genetic Analyzer 3100 (Thermo Fisher Scientific, Inc.) by an external supplier, the Instituto Potosino de Investigación Científica y Tecnológica (IPICYT, San Luis Potosí, Mexico). Sequences were analyzed with CodonCode Aligner software (CodonCode Corp., Centerville, MA, USA). The obtained recombination sequence was the same as the one previously published in 2007 when the *BRCA1* ex9-12del mutation was reported (15).

Design of the qPCR primers and TaqMan® probes. Using the recombination sequence, primers were designed to

amplify a 164 bp fragment flanking this recombination site. Therefore, the F primer was designed to bind to a sequence in intron 8, and the R primer to intron 12. Melting temperature of both primers was 60°C. Primers were tested in samples with the *BRCA1* ex9-12del mutation, resulting in the amplification of a single 164 bp DNA fragment. The TaqMan® probe complementary to the recombination region was designed using the software Custom qPCR Probes from Integrated DNA Technologies (IDT, Coralville, IA, USA). The probe was ordered with FAM™ dye and the quenchers ZEN™/IB®FQ.

As a DNA control, a PCR was included to amplify a region of exon 11 from *BRCA1*. Design of primers and TaqMan® probe specific for exon 11 was carried out using the reference sequence (L78833.1) the same way as for the *BRCA1* ex9-12del mutation, but HEX™ dye was used instead.

qPCR with designed primers and TaqMan® probes. Thermocycler StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific, Inc.) was used to perform the qPCR with TaqMan® probes. The PCR was prepared with 1X TaqMan® Universal PCR Master Mix (Thermo Fisher Scientific, Inc.), 0.2 μM primer F, 0.2 μM primer R, 250 nM probe, 10 ng DNA, and nuclease-free water to a total volume of 15 μl. Thermal cycling conditions were as follows: 60°C for 30 sec, 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 sec and 60°C for 1 min. A final post-read method was set

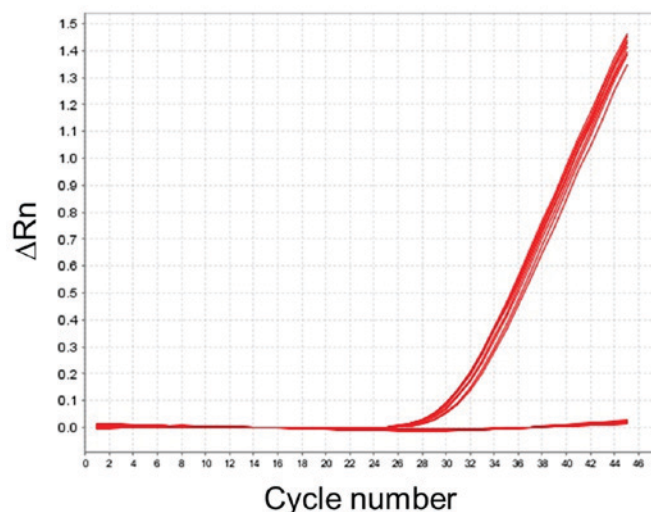


Figure 2. Detection of the *BRCA1* ex9-12del mutation by real-time PCR and TaqMan® probe. Red amplification curves represent the fluorescent signal given by the FAM™ dye from the TaqMan® probe detecting the variant allele in the four positive samples, which had a mean Ct of 27.46. No amplification curve is observed for the negative samples neither for negative controls (horizontal red lines). The 'Y' axis values represent the fluorescent signal normalized of the FAM™ dye with the ROX, whereas the 'X' axis represent number of cycles. PCR, polymerase chain reaction; ROX, passive reference dye.

at 60°C for 30 sec. The passive reference dye included in the TaqMan® Universal Master Mix was ROX.

Analysis of results. The fluorescent signal of the TaqMan® qPCR was analyzed using the StepOnePlus™ software v2.3 (Thermo Fisher Scientific, Inc.) under presence/absence and genotyping methods. In the presence/absence analysis, a positive result for a deleterious mutation (*BRCA1* ex9-12del) was the amplification signal of the recombination region given by the probe carrying the FAM™ dye, displayed in an amplification plot. We considered a positive signal at a Ct between 27 and 27.5. As a control we used the amplification signal of the exon 11 given by the probe carrying the HEX™ dye.

TaqMan® Genotyper software was used to analyze raw data from qPCR in an allelic discrimination (AD) plot. The regular AD plot shows each sample well as an individual dot. The AD plot can show different clusters of dots such as homozygous and heterozygous samples, no-template controls (NTC) and undetermined samples. The dots of each cluster are grouped closely and each cluster is located far from the others. In a typical AD plot, the cluster of homozygous samples for allele 1 labeled with HEX™ dye is located in the lower right corner. The cluster of homozygous samples for the allele 2 labeled with FAM™ dye is located in the upper left corner and the cluster of heterozygous (allele 1/allele 2) samples is located approximately midway between both homozygous clusters. NTC samples are located in the lower left corner. The undetermined samples are represented by an X and are located in any region of the AD plot outside of the described above. Nonetheless, the clusters displayed in the AD plot from our TaqMan® qPCR assay are different, as explained below. Carriers of at least a single copy of the wild-type allele (exon 11) are located in the lower right corner (HEX™). Carriers of at least one allele with

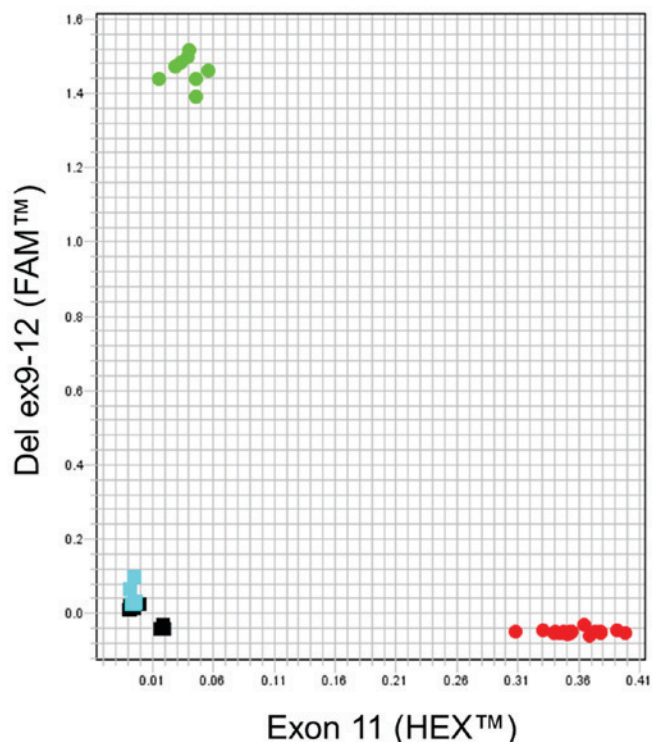


Figure 3. Genotyping assay for the detection of the mutant allele (with the *BRCA1* ex9-12del mutation) and the wild-type allele (exon 11) by real-time PCR and TaqMan® probes. The AD plot shows 2 groups; green color samples have the allele with the deletion, samples in red color possess exon 11 or the wild-type allele (in this group all the samples are present, the 6 negative and the 4 positive ones). Samples in blue color are negative samples for the deletion, which have no fluorescence and are classified by the software as negatives. Negative controls are shown in black squares. Each sample was processed by duplicate. The axis values represent relative fluorescence (ΔRn) between both dyes (FAM™ for the mutant allele and HEX™ for the wild-type allele). PCR, polymerase chain reaction; AD, allelic discrimination.

the *BRCA1* ex9-12del mutation are clustered in the upper left corner (FAM™).

Results

DNA samples. A total of ten DNA samples were subjected to different experiments in order to detect the *BRCA1* ex9-12del mutation. Four of them were previously known as carriers of the *BRCA1* ex9-12del mutation, three obtained from females and one from a male. Three of these samples of mutation carriers were family members (mother, daughter and son) and one from an unrelated female. Samples used as controls were obtained from six unrelated females not suspected to carry the *BRCA1* ex9-12del mutation.

End-point PCR. Positive and negative samples of the *BRCA1* ex9-12del mutation were analyzed with the end-point PCR method. A unique band of ~630 bp was observed in the agarose gel with primers F and R2 in the four positive samples. This band was not observed in the six negative samples (Fig. 1).

qPCR with TaqMan® probes using the presence/absence method. The thermocycler allows performing a PCR under different types of methods, such as presence/absence, genotyping and relative standard curve. The positive and negative

Table I. Comparison between qPCR, end-point PCR and MLPA techniques for the detection of the *BRCA1* ex9-12del mutation (15,31,36,37,38).

Variable	qPCR and TaqMan [®] probes	End-point PCR	MLPA
Required equipment	Real-time PCR thermocycler	Conventional PCR thermocycler, electrophoresis chambers, electrophoresis power supply, microwave oven and UV transilluminator to visualize agarose gels.	Conventional PCR thermocycler and capillary sequencer.
Amount of DNA required per reaction	2.5 ng	≥80 ng	50-250 ng
Processing time until results (excluding DNA extraction)	2 h	3.5 h	24 h
Sample number to be simultaneously processed (fitting to the processing time)	90	25	96

qPCR, quantitative polymerase chain reaction; MLPA, multiplex ligation-dependent probe amplification; UV, ultraviolet.

samples of the *BRCA1* ex9-12del mutation were analyzed with qPCR and the TaqMan[®] probe complementary to the recombination region under the presence/absence method. Four DNA samples showed amplification signal from FAM[™] dye, indicating the presence of the *BRCA1* ex9-12del mutation. These positive samples showed amplification curves at Ct mean 27.46 (Fig. 2). This signal was absent in the six control samples.

qPCR with TaqMan[®] probes using the genotyping method. In order to identify heterozygous from homozygous carriers in a single analysis, the ten DNA samples were tested by the genotyping method. Six subjects showed fluorescent signal only from HEX[™] dye, meaning they carry only the wild-type allele (exon 11). In the AD plot, these samples are located in a single cluster in the lower right corner (Fig. 3). Four subjects showed both fluorescent signals, from HEX[™] and FAM[™] dyes, indicating the presence of exon 11 and the *BRCA1* ex9-12del mutation, respectively. Thereby, these are heterozygous samples which were located in two clusters, one corresponding to the HEX[™] signal (lower right corner) and one to the FAM[™] signal (duplicates in the upper left corner) (Fig. 3).

Discussion

Currently, different methods are used for the early detection of cancer, such as analysis of circulating tumor cells (CTCs), as well as RNA exosomes (27), proteins (3) and genomic profiles of circulating cell-free tumor DNA (ctDNA) from liquid biopsy (27,28). Although the application of these methods can be useful to improve prognosis, there are still some limitations (28). For example, they are more effective for early detection of certain types of cancer, such as ovarian cancer, but less effective to detect breast cancer (29). Therefore, there are no well-defined biomarkers for all types of cancer (28). However, the effectiveness of breast cancer detection can be improved by including tests that identify mutations in the *BRCA* genes (30).

The inclusion of *BRCA* screening in the health care system is highly affected by the cost of *BRCA* testing (30). Therefore, it would be desirable to have alternative low-cost options for the detection of mutations. The proposed method for the detection of the *BRCA1* ex9-12del mutation is based on qPCR and TaqMan[®] probes. This method was capable of detecting four positive *BRCA1* ex9-12del mutation carriers, which were previously analyzed by our end-point PCR method. This was the expected result, since the probe was designed to be complementary to the mutant allele, specifically in the recombination region between introns 8 and 12, which is a unique sequence in the human DNA.

Among the main advantages of this method is the low cost, which is 2-3 USD per sample, compared to a price of ~500 USD for the determination of a specific mutation. High prices are a limitation for those who wish to know their genetic status regarding *BRCA1* ex9-12del mutation. Using qPCR allows high sensitivity since the reaction can be carried out only with 2.5 ng of DNA (31). In addition, a TaqMan[®] probe allows high specificity since it binds only to the sequence to which is complementary, even if a single base pair differs from the probe and the DNA region. The fact that a small amount of DNA is required for the assay gives an extra strength to this method, since DNA can be extracted from small biological samples taken in a non-invasive manner, such as urine, epithelial cells from the mouth or hair roots. Peripheral blood guarantees a high DNA yield, but its extraction is painful and uncomfortable for the patient, especially when the quality of the veins is not good due to prior chemotherapy. It is a fast method since in 2 h a total of 90 samples can be analyzed in a single experiment, including the interpretation.

Biallelic mutations in both *BRCA* genes are rare (32,33). Therefore, deleterious mutations in these genes are mainly heterozygous (34). However, biallelic pathogenic mutations have been reported in individuals with ovarian cancer. Nevertheless, those patients are compound heterozygous, this is, they carry mutations in both *BRCA1* copies, but in different regions (for example: c.2457delC/c.5207T>C) (33). Biallelic

mutations in *BRCA* genes could increase the risk of cancer, as well as leading to more severe phenotypes (32,35). To date, no individual has been found homozygous for the *BRCA1* ex9-12del mutation. Most probably the latter would not be compatible with life since great quantity of genetic material is lost. However, it is important to detect any region of the wild-type allele at the same time that we detect the mutant allele. This is also helpful as a control of DNA presence in the sample. Consequently, we also designed an assay based on qPCR and a TaqMan® probe to detect a region from exon 11 of *BRCA1*.

When performing a TaqMan® qPCR run under the genotyping method, the amplification of both variant and wild-type alleles is ideally carried out in the same reaction well; this is, in a multiplex fashion. Nonetheless, in our study, we performed the PCR of the variant allele and the PCR of the wild-type allele in separate reaction wells. So, a sample will be defined as heterozygous only if it appears in both clusters in the AD plot (Fig. 3). Consequently, as future study, we aim to standardize a multiplex qPCR capable of detecting the variant allele (recombination region) and the wild-type allele (a region from exons 9 to 12) in one single reaction, saving time and reagents.

Since *BRCA1* ex9-12del mutation is considered as a founder mutation, and the existing reports have shown that the recombination region is the same among all carriers of this mutation; our method is considered highly specific because the TaqMan® probe binds to this recombination DNA sequence. However, if a different recombination region is discovered, or if any additional mutations arise in this region, our method would have to be modified accordingly and readapted to the newly reported genetic variants.

Compared to the reported methods used to detect the *BRCA1* ex9-12del mutation, our propose represents a suitable option for testing given the advantages already mentioned.

The software of the qPCR thermocycler analyzes the results at the end of the run, displaying the presence or absence of the mutation and therefore, making the interpretation an easy step. Moreover, working with qPCR avoids the risk of post-amplification contamination, since only one process is needed. On the other hand, end-point PCR requires a first step of amplification, followed by the run of the samples on an agarose gel, a process in which the samples can be cross-contaminated (36). Comparing to the PCR method reported by Weitzel *et al.*, in 2007 (15), our design requires less DNA input, 2.5 ng (31), whereas the Weitzel method requires 50-100 ng. Additionally, it does not require further manipulation of the sample, for example purification of the amplified product, sequencing-PCR and capillary electrophoresis. The analysis of results is faster and the identification of the heterozygous carriers is an easier task (15). Another method used to detect deletions is MLPA, which has the great advantage of detecting more than one mutation since it analyzes different regions of the genome simultaneously. However, for the detection of the *BRCA1* ex9-12del mutation, this method has the disadvantage of requiring a large amount of DNA input (250-450 ng), increased sample handling, carrying an additional risk of cross-contamination. It also requires capillary sequencer and the analysis is more complex (37) compared to our TaqMan® real-time method. A comparison between

current methods for the detection of the *BRCA1* ex9-12del mutation is summarized in Table I.

The method we propose will allow the determination of a high number of samples at the same time, which will be a great advantage in future studies of association or prevalence. Additionally, it is a safe, specific and more affordable option for those wishing to be tested for the mutation.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

DAMT designed the sequences of primers and probes, performed DNA isolation, PCR experiments and wrote the manuscript. RBRLC conceived the study, directed, planned and analyzed the data and wrote manuscript. CVG and DAM performed the participants' selection, clinical evaluation and interpreted the results. EAM and HABS analyzed the data, interpreted the results and critically reviewed the manuscript.

Ethics approval and consent to participate

The protocol was approved by Ethics, Research and Biosecurity Committees of the University of Monterrey, San Pedro Garza Garcia, Nuevo Leon, Mexico, registry no. 01072017-CIE. Written informed consent was obtained from all subjects.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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