**ORIGINAL ARTICLE**

# *BRCA1* and *BRCA2* screening of nine Chilean founder mutations through allelic-discrimination and real-time PCR in breast/ovarian cancer patients

Carolina Alvarez[1] · Victoria Ortega-Hernández[1] · Aracely Cortez[1] · Pilar Carvallo[1]

## Abstract

**Background** In a previous work, we identified nine founder mutations present in close to 80% of *BRCA1* and *BRCA2* mutation carriers, and distributed across the country. The presence of founder mutations constitutes a valuable opportunity to develop new strategies for genetic screening. Genetic tests are primarily performed by NGS sequencing, which requires sophisticated and expensive equipment, and it takes 2–3 weeks for the results to be informed to the patient. In addition, genetic tests are not covered by insurance companies in Latin American countries. In this work, we present the standardization and technical validation of a real-time PCR based methodology for allelic discrimination in order to identify the nine Chilean founder mutations in *BRCA1* and *BRCA2* genes.

**Methods and results** We designed nine pairs of probes and nine pairs of primers to amplify synchronically nine regions of the *BRCA1/BRCA2* genes by real-time PCR, in order to identify the nine founder mutations through allelic discrimination analyses. Technical validation was performed using 90 positive and 90 negative samples for each mutation. The methodology was tested in a second group of 60 patients. Our method correctly classified carriers and non-carriers of one of the nine Chilean founder mutations with a 100% specificity and 100% sensitivity, compared with Sanger sequencing performance.

**Conclusions** We develop an inexpensive, simple, and fast mutation detection method that could be implemented locally in Hospitals from the Private to Public health system. This methodology may be useful for the screening of *BRCA1* and *BRCA2* mutations in other populations.

**Keywords** BRCA1 · BRCA2 · Founder mutation · Real-time PCR · Mutation screening · Fluorescent probes

## Introduction

Mutations in *BRCA1* and *BRCA2* confer an accumulative risk of 52–57% and 47–49% to develop breast cancer before age 70, respectively [1, 2]. In the early 90s, when the identification of those genes was in progress, it was defined that hereditary breast cancer represented 5–10% of the total breast cancer cases [3, 4]. By that time, it only considered families with a strong history of breast and/or ovarian cancer in first-degree relatives and with complete penetrance. As mutation screening became more frequent,

it was found that most families with breast and/or ovarian cancer had second- and third-degree relatives affected and presented incomplete penetrance. This second group was classified as familial breast cancer and represents 15–20% of the total breast cancer [5, 6]. Nowadays, genetic testing of *BRCA1* and *BRCA2* mutations is recommended additionally to patients with no apparent family history, but diagnosed at early age (under 50), having bilateral breast cancer or triple negative breast cancer under 60 years of age, among other criteria, according to the guidelines of The National Comprehensive Cancer Network (NCCN) [7]. The extension of inclusion criteria opens the need to develop less expensive mutation detection strategies to support the genetic testing of a greater group of patients.

An extensive study performed by our group in 453 Chilean breast and/or ovarian cancer patients describes the finding of nine founder mutations [8] present in close to 80% of all *BRCA1* and *BRCA2* mutation carriers, distributed across

✉ Carolina Alvarez
csalvare@bio.puc.cl

1 Laboratorio de Genética Molecular Humana, Departamento de Biología Celular y Molecular, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Portugal 49, 3rd floor, 8330025 Santiago, Chile

the country. The percentage of carriers of a founder mutation in *BRCA1* and *BRCA2* is higher in families whose ancestors came to Chile during Spanish colonization (1540–1750). These families are attended today, mainly in the Public Hospitals, nevertheless in the private system we have detected that the prevalence of these mutations is still high (55%). The reason for the lower percentage in the private system is the higher proportion of more recent immigrants within this group of patients. The presence of founder mutations in a high proportion of Chilean patients gave us the opportunity to develop an inexpensive, simple, and fast method to detect these mutations.

Today, detection of *BRCA1* and *BRCA2* mutations in breast and ovarian cancer patients is highly demanded. Genetic tests are primarily performed by NGS sequencing, but this technology requires sophisticated and expensive equipment and it takes 2–3 weeks for the results to be informed to the patient [9]. In addition, genetic tests for breast and ovarian cancer patients, offered mainly by providers in Europe and North America, are covered by insurance companies in these regions of the world. This is not the situation for developing countries, such as Latin Americans and particularly Chile, in which patients have to pay for their genetic tests. For this reason, it becomes evident the need to develop new methodologies that could be performed in situ, allowing a simpler, faster, inexpensive genetic screening.

In the last 10–15 years, different methodologies have been proposed for specific *BRCA1* and *BRCA2* mutation detection. Among these methods are enhanced mismatch mutation analysis (EMMA), enzyme mismatch cleavage (EMC), high resolution melting (HRM) and probe-based genotyping [10–13]. Real-time PCR based mutation detection have been used for allelic discrimination assays either using allele specific primers, HRM or fluorescent probes. It is worth mentioning that the implementation of real-time PCR for the mutational screening in other genes has been successful, with high sensitivity and specificity [14–16]. The predilection of this technique for genetic screening is given because it is faster, simpler and less expensive compared with NGS or even the gold standard, Sanger sequencing. Additionally, after COVID-19 PCR detection was implemented, most molecular laboratories in health institutions are trained in real-time PCR.

Here we describe the standardization and technical validation of a real-time PCR-based protocol that allows discriminating between normal (homozygous) DNA and DNA samples from mutation carriers (heterozygous), using fluorescent probes. Due to the low cost, simplicity and fast implementation of our methodology, this protocol could be potentially used for extensive, population-based screening of the nine founder mutations in *BRCA1* and *BRCA2* in Chilean breast cancer patients.

## Results

Method standardization was performed in at least six independent experiments using a heterozygous sample with the specific mutation, a normal homozygous control sample and a non-template control, all in triplicates. Figure 1 shows allelic discrimination plots of standardization experiments for all mutations. As shown, all heterozygous samples presented FAM and VIC fluorescence while normal homozygous samples emitted only VIC fluorescence as expected. Average fluorescence detected for each probe in normal control and heterozygous samples is shown in Table 1. As observed for each mutation, there are differentiable values between the normal homozygous DNA samples and heterozygous DNA samples, having a distinct spatial location in allelic discrimination plots (Fig. 1). These results show a good discrimination power of the designed probes for each mutation. The standard deviations for the observed fluorescence intensities with each probe in DNA samples varied between 2 and 19% in relation to the average fluorescence intensity obtained with the different probes, an acceptable variability among replicates.

After PCR conditions were standardized, we technically validated the mutation detection using a set of 90 blind samples per mutation as described in methods. Technical validation was performed by two different laboratories, testing a total of 180 samples per mutation. Figure 2 shows allelic discrimination plots obtained for technical validation of each mutation for 90 blind samples. As shown, this validation was successful, allowing us to clearly classify all mutation carriers over controls. Supplementary Table 2 summarizes average fluorescence obtained for each mutation in the technical validation assay for mutated and control samples. As shown, standard deviations varied between 2 and 18% confirming standardization results. Table 2 summarizes technical validation results in terms of sample assignment as positive or negative for a specific mutation. The cells label as positive correspond to samples showing heterozygous genotype in the allelic discrimination plot, whereas the cells label as negative are samples showing normal homozygous genotype. No false negatives or false positives were observed since no carrier sample was negative for its mutation, and no normal control DNA was classified as positive. These results allowed us to determine a 100% sensitivity and 100% specificity for the detection of each mutation independently, and for the whole assay.

We then apply our detection method in a second sample set of 60, non-previously studied, in a relevant environment context for genetic screening. These patients were analyzed in groups of variable numbers of samples, as they were recruited. In this opportunity, we used our protocol to screen the nine founder mutations in all

**Fig. 1** Allelic discrimination plots obtained from standardization of real time PCR assays for each founder mutation. Each plot shows FAM fluorescence (Y axis) and VIC fluorescence for normal probes (X axis). Each assay was performed with triplicates of a mutated sample, a control DNA and a no template control. Genotype calls are indicated in the chart on the right

**Table 1** Average fluorescence intensities observed with each probe in the standardization of the allelic discrimination assays for the nine *BRCA1* and *BRCA2* founder mutations

| Analyzed founder mutations | Sample | Fluorescence intensity ($\Delta$Rn) | |
| --- | --- | --- | --- |
| | | VIC | FAM |
| BRCA1 | | | |
| c.1504_1507delTTAA | No template control | $0.201 \pm 0.2$ | $0.227 \pm 0.11$ |
| | Control DNA | $1.431 \pm 0.05$ | $0.088 \pm 0.03$ |
| | Mutated DNA | $0.672 \pm 0.09$ | $2.656 \pm 0.14$ |
| c.3331_3334del CAAG | No template control | $0.157 \pm 0.05$ | $0.23 \pm 0.09$ |
| | Control DNA | $0.985 \pm 0.09$ | $1.237 \pm 0.21$ |
| | Mutated DNA | $0.413 \pm 0.08$ | $3.184 \pm 0.16$ |
| c.3759dupT | No template control | $0.423 \pm 0.17$ | $0.185 \pm 0.08$ |
| | Control DNA | $1.212 \pm 0.04$ | $0.363 \pm 0.03$ |
| | Mutated DNA | $0.743 \pm 0.03$ | $2.450 \pm 0.1$ |
| c.3817C>T | No template control | $0.514 \pm 0.18$ | $0.05 \pm 0.07$ |
| | Control DNA | $1.801 \pm 0.06$ | $0.238 \pm 0.08$ |
| | Mutated DNA | $1.102 \pm 0.09$ | $2.441 \pm 0.37$ |
| BRCA2 | | | |
| c.145G>T | No template control | $0.456 \pm 0.00$ | $0.177 \pm 0.08$ |
| | Control DNA | $1.101 \pm 0.05$ | $0.032 \pm 0.02$ |
| | Mutated DNA | $0.663 \pm 0.04$ | $1.599 \pm 0.03$ |
| c.4740_4741dupTG | No template control | $0.049 \pm 0.01$ | $0.158 \pm 0.03$ |
| | Control DNA | $0.728 \pm 0.02$ | $0.104 \pm 0.01$ |
| | Mutated DNA | $0.426 \pm 0.02$ | $1.609 \pm 0.06$ |
| c.5146_5149delTATG | No template control | $0.115 \pm 0.02$ | $0.331 \pm 0.03$ |
| | Control DNA | $0.466 \pm 0.01$ | $0.272 \pm 0.01$ |
| | Mutated DNA | $0.297 \pm 0.00$ | $2.070 \pm 0.14$ |
| c.8987T>A | No template control | $0.641 \pm 0.19$ | $0.251 \pm 0.11$ |
| | Control DNA | $1.378 \pm 0.08$ | $0.424 \pm 0.10$ |
| | Mutated DNA | $1.111 \pm 0.06$ | $2.448 \pm 0.21$ |
| c.9382C>T | No template control | $0.234 \pm 0.16$ | $0.211 \pm 0.15$ |
| | Control DNA | $1.338 \pm 0.08$ | $0.007 \pm 0.03$ |
| | Mutated DNA | $0.732 \pm 0.05$ | $0.612 \pm 0.06$ |

new patients. As described, all samples were Sanger sequenced and results were kept confidential until the nine founder mutations were analyzed by real-time PCR. Figure 3 shows results obtained in this validation set. Six patients (FID6, FID12, FID26, FID39, FID43 and FID45) were detected as carriers of one of the nine founder mutations using our real-time PCR-based methodology, and confirmed by Sanger results. In addition, three relatives of FID39 (FID39-4, FID39-6 and FID39-7) were detected positive for the familial mutation. The results with the new DNA samples confirmed a specificity and sensitivity of 100% of our methodology.

## Discussion

In this work, we show the development of a simple, inexpensive and fast real-time PCR based protocol for allelic discrimination in order to identify nine Chilean founder mutations in *BRCA1* and *BRCA2* genes. The protocol was developed and standardized using a training sample set from previously identified mutation carriers, technically validated afterwards with a blinded set of 90 samples of known *BRCA1* and *BRCA2* mutation status, and followed by the screening of the nine founder mutations in a final set of 60 newly recruited patients. The protocol is based on the use of fluorescent probes specific for the normal or the mutated allele, labeled with VIC or FAM, respectively.
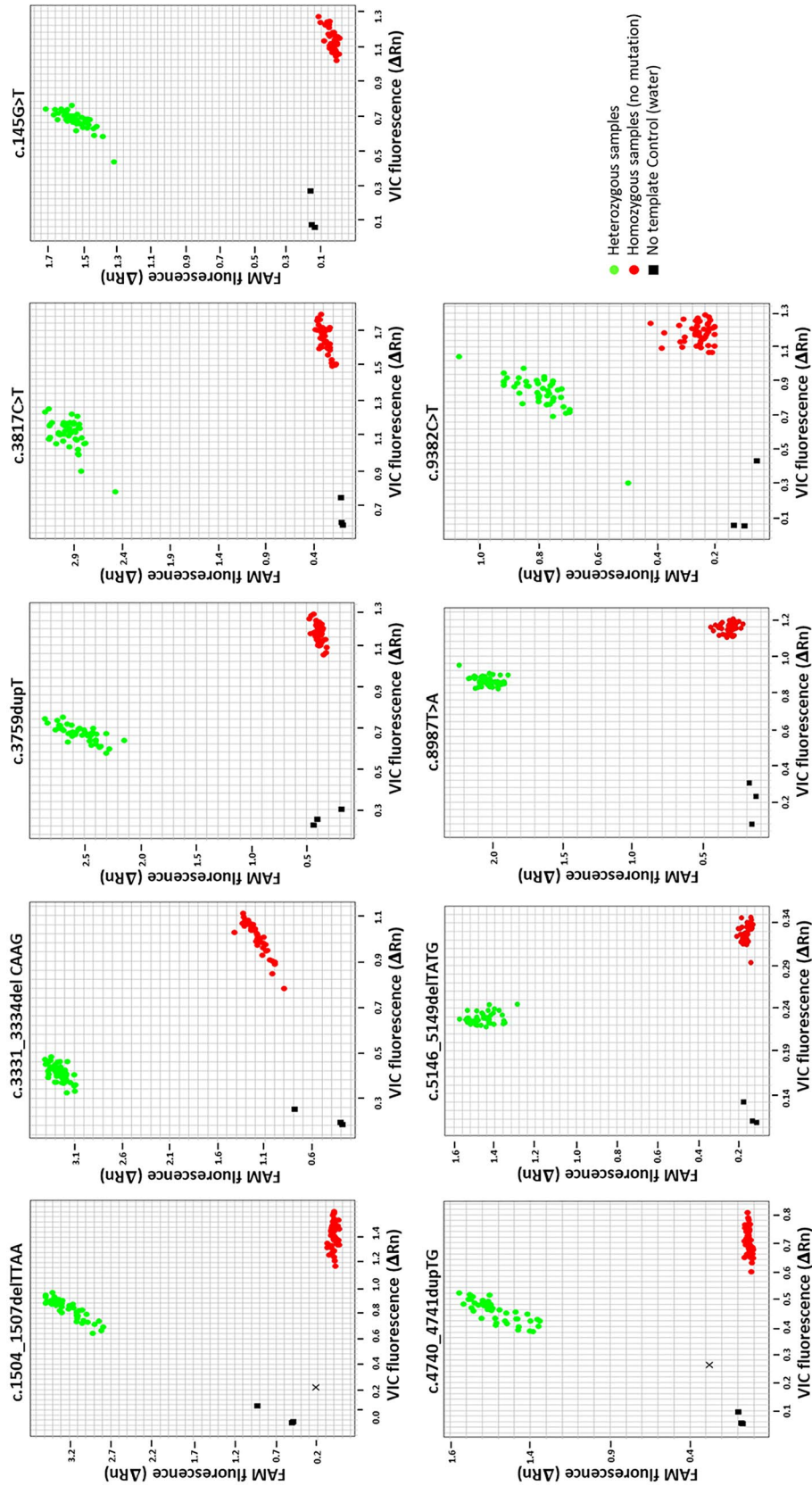
**Fig. 2** Allelic discrimination plots obtained from the validation of real time PCR assays for each founder mutation. Each plot shows FAM fluorescence for mutated probes (Y axis) and VIC fluorescence for normal probes (X axis). Each assay was performed with a set of 90 blinded randomized samples (45 mutated and 45 control DNA samples) and a triplicate of a no template control. Genotype calls are indicated in the chart on the right. All samples successfully amplified were correctly genotyped in the assays

**Table 2** Summary of technical validation results showing samples assigned as positive or negative for each of the nine founder mutations

| Mutation | | Results | Nº carriers (patients) | Nº non carriers (controls) |
|---|---|---|---|---|
| BRCA1 | c.1504_1507delTTAA | Positive | 90 | 0 |
| | | Negative | 0 | 89 |
| | c.3331_3334delCAAG | Positive | 89 | 0 |
| | | Negative | 0 | 89 |
| | c.3759dupT | Positive | 84 | 0 |
| | | Negative | 0 | 87 |
| | c.3817C>T | Positive | 85 | 0 |
| | | Negative | 0 | 87 |
| BRCA2 | c.145G>T | Positive | 83 | 0 |
| | | Negative | 0 | 86 |
| | c.4740_4741dupTG | Positive | 79 | 0 |
| | | Negative | 0 | 85 |
| | c.5146_5149delTATG | Positive | 90 | 0 |
| | | Negative | 0 | 90 |
| | c.8987T>A | Positive | 90 | 0 |
| | | Negative | 0 | 90 |
| | c.9382C>T | Positive | 88 | 0 |
| | | Negative | 0 | 88 |

We observed different fluorescence intensity values according to the analyzed mutation. This is due to several parameters of the PCR reaction: the probe sequence, the characteristics of the mutation and the position of the mutation site within the probe. The analyses for the detection of each mutation reveal that the discrimination power for each pair of probes permits to differentiate with no error the normal and mutated DNA samples. This is evidenced through the generation of two separate samples clusters in the allelic discrimination plots allowing the correct calling of the genotypes in every assay. For this reason, during the technical validation step we were able to correctly identify carriers and non-carriers of one of the nine founder mutations in *BRCA1* and *BRCA2* with a 100% specificity and 100% sensitivity, contrasted with the mutation detection by Sanger sequencing.

After technical validation, the protocol was used to screen the nine founder mutations in 60 newly recruited patients including some healthy relatives at risk. We were able to determine the presence of five of the nine mutations in a total of nine samples, showing 100% concordance with Sanger sequencing results. Our results show the outstanding performance of our mutation detection method in a relevant environment, which is the analysis of new patients consulting for genetic testing in *BRCA1* and *BRCA2* genes.

Today different genetic services in Europe and North America offer genetic tests for breast and/or ovarian cancer patients, which are covered by insurance companies in these regions of the world. This is not the situation in developing countries, as Latin Americans and particularly in Chile, in which patients have to pay for their genetic tests. The direct cost of the test described here is close to USD30 per patient, with the great advantage of implementing the methodology in situ, in clinical laboratories from the public to private system. In addition, the cost for one mutation detection in family members at risk is also decreased. In conclusion, our methodology will serve as genetic test for breast and/or ovarian cancer patients in the private system and will give an opportunity to patients in the public system to have access to *BRCA1* and *BRCA2* genetic screening.

In addition to our findings in Chile, there are a few mutations with a demonstrated founder effect in Mexico, Brazil and Colombia [17, 18]. The genetic test described here may be useful for a genetic screening in these populations or others. For example, a similar test using TaqMan probes has been reported in Mexico for one of the founder mutations in this population, which is the *BRCA1* exon9-12del [13].

Expanding genetic diagnostics to a larger group of breast and/or ovarian cancer patients, in developing countries, will give them the opportunity to receive a more specific therapy leading to a better outcome. To their relatives at risk it will permit them to improve prevention strategies, such as prophylactic surgeries or clinical follow up for an early diagnosis. In addition, increasing genetic testing will improve knowledge in relation to clinical and pathological features of women carrying a *BRCA1* or a *BRCA2* mutation in Chile,
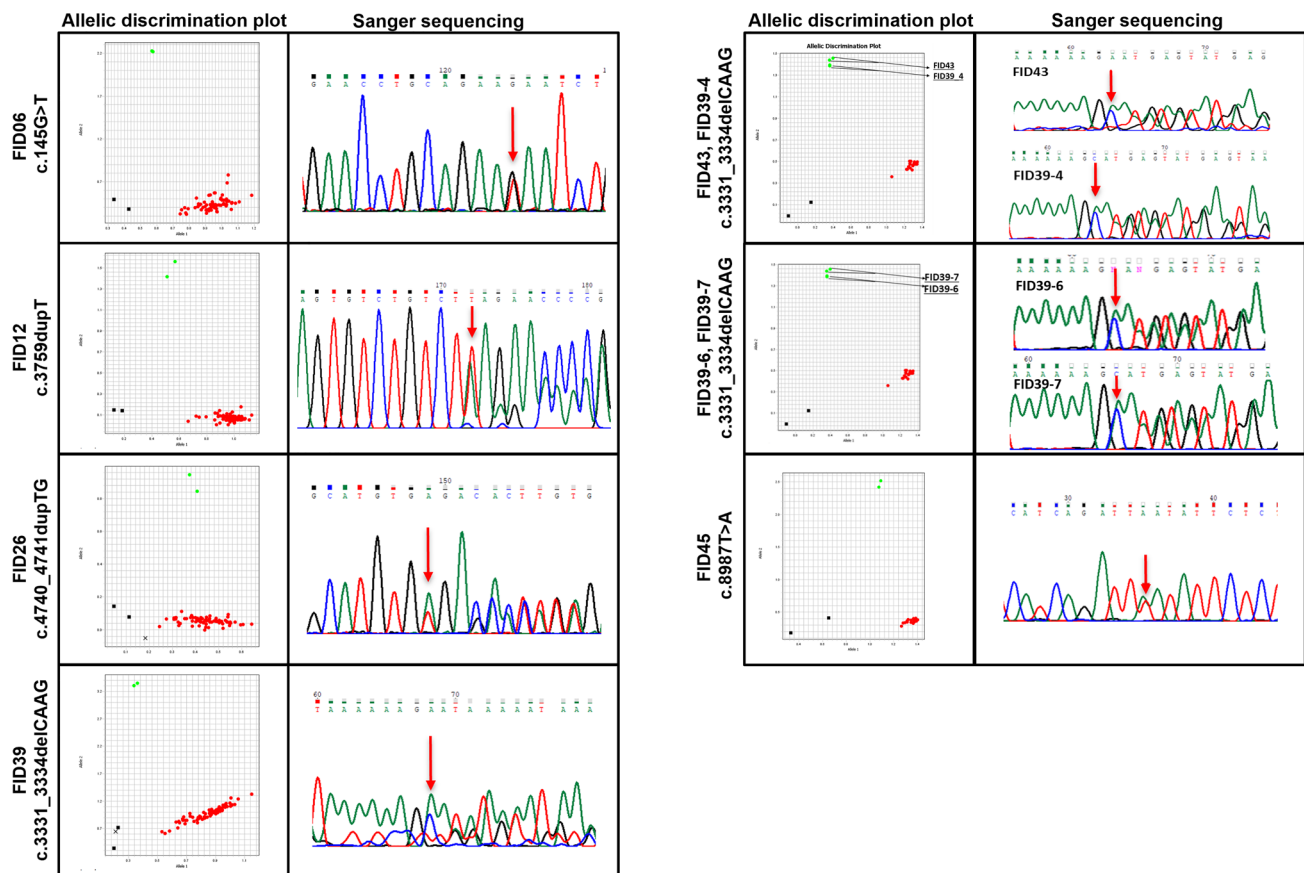
**Fig. 3** Genotyping results obtained for patients with mutations detected by our probed-based real-time PCR protocol and Sanger sequencing. As shown, all samples identified through allelic discrimi-nation assays as carriers of one of the BRCA1 and BRCA2 founder mutations were confirmed by Sanger results. The arrows indicate position of the mutation in the electropherograms

and to implement specific therapeutic strategies in breast cancer patients carrying a *BRCA1* or a *BRCA2* mutation.

## Materials and methods

### Patients and DNA samples

All samples were obtained from patients meeting one of the following criteria: (1) three relatives with breast cancer at any age, (2) two relatives with breast cancer, one diagnosed before age 45, (3) two relatives with cancer, one with breast and one with ovarian cancer and (4) two relatives with breast cancer, one of them being a man. In addition, we selected patients with no family history, but that (1) presented bilateral breast cancer or (2) developed breast cancer before age 40. All patients signed an informed consent approved by the Scientific-Ethics Committee of the Pontificia Universidad Católica de Chile.

Method standardization was performed using DNA samples with a known *BRCA1* and *BRCA2* mutation. These samples came from patients recruited between 2000 and 2015 and their mutation identification was assessed by Sanger. DNA samples with mutations were used firstly as positive controls to determine the optimal conditions for mutation detection, and secondly, as a blind set of samples randomized with negative controls for the technical validation of the methodology. Technical validation was performed in two separate laboratories at Hospital Base de Valdivia and Pontificia Universidad Católica de Chile. A second group of 60 samples was included during the study from two hospital centers in Chile: Hospital Base de Valdivia and Hospital Clinico UC Christus. Genomic DNA was isolated from peripheral blood using the method described by Lahiri and Nurnberger [19] and stored at 4 °C. The presence of the nine founder mutations was determined in all DNA samples using real-time PCR-based methodology (described below), and Sanger sequencing

(see Supplementary Table 1 for primers used for Sanger). All results obtained with the real-time PCR-based methodology were compared to sequencing results in order to determine the specificity and sensitivity.

## Primers and probes

To evaluate the presence of the nine founder mutations we designed nine pairs of primers and nine pairs of probes. Primers were designed using PrimerBLAST (NCBI) to generate products within a size range of 140–180 bp and were located as equidistant to each mutation site as possible. All primers have a Tm close to 61 °C. Probes were designed to have a length between 15 and 30 bp and the mutation site located at the center or displaced slightly to the 5′ of the probe. We placed between 4 and 6 LNA modifications in the probes, especially in nucleotides involved in the mutations and/or adjacent to them in order to improve binding strength and achieve a Tm over 65 °C. All probes recognizing mutated alleles were labeled with 6-FAM, and all probes for normal alleles were labeled with HEX, read as VIC by the real-time PCR system. Hairpin, homoduplex and heteroduplex formation for all oligonucleotides were evaluated using Oligoanalyzer (Integrated DNA Technologies), and unspecific hybridization was analyzed with Primer Blast (NCBI). All the sequences for primers and probes are included in a patent under revision (register number 2020003418).

## Real time PCR

PCR was performed in 20 μl reactions containing 10 ng of DNA, 250 nM of each probe (5 pmol each) (for mutation 2: 500 nM for normal allele and 75 nM for mutated allele targeted probes, respectively), 500 nM of each primer (10 pmol each) and 1× HOTFIREPol PROBE qPCR Mix Plus (ROX) (Solis BioDyne, Estonia). The PCR program consisted of 30 s of initial reading at 60 °C, enzyme activation for 15 min at 95 °C, 40 cycles of 30 s at 95 °C and 1 min at 61 °C, and a final reading at 60 °C for 30 s. All PCR reactions were performed in a QuantStudio 3 real-time PCR system (Applied Biosystems, Thermo Fisher Scientific, USA). Results were analyzed in QuantStudio™ Design and Analysis Software v1.5.1 (Applied Biosystems, Thermo Fisher Scientific, USA) using allelic discrimination cartesian plot for genotype calls. Supplementary Figure S1 shows a representation of the allelic discrimination assays using real-time PCR with fluorescent probes. All mutations were analyzed simultaneously in a single assay under the described PCR program.

## Specificity and sensitivity

Calculations were performed as follows: Eq. (1) for sensitivity and Eq. (2) for specificity.

$$Sensitivity = \frac{true\ positive}{(true\ positive + false\ negative)} \tag{1}$$

$$Specificity = \frac{true\ negative}{(false\ positive + true\ negative)} \tag{2}$$

where true positive: a patient *carrying* a specific mutation, resulting *positive* for such mutation in the validation assay. True negative: a patient *not carrying* a specific mutation, resulting *negative* for such mutation in the validation assay. False positive: a patient *not carrying* a specific mutation, resulting *positive* for such mutation in the validation assay. False negative: a patient *carrying* a specific mutation, resulting *negative* for such mutation in the validation assay.

## Declarations

**Conflict of interest** The authors have no relevant financial or non-financial interests to disclose.

**Ethical approval** This study was approved by the Scientific-Ethics Committee of the Pontificia Universidad Católica de Chile. All patients signed an informed consent previous to the sample collection. Patients were informed of the study, its benefits and probable risks, and their participation was voluntary and confidential.

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