

Identification of Germinal Mutations in Eight Exons of the *BRCA1* Gene in Breast Cancer Patients in the State of Amazonas, Using Direct Sequencing

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Abstract: Mutations in the *BRCA1* gene are responsible for 50% of cases of hereditary breast cancer. The objective of this study was to perform screening of mutations in the *BRCA1* gene in breast cancer patients treated in the state of Amazonas, Brazil. We analyzed 53 patients (51 women and 2 men) ranging from 30 to 71 years of age. Most of these patients were born in the state of Amazonas, and had a family history for predisposition to cancer and evidenced hormonal risk factor. The alterations found in the exons of the gene that were studied were verified in three online databases for this gene (ClinVar, BRCA Exchange and Varsome). A total of four mutations were identified: missense mutation c.4304T > C exon 13 (16 patients), missense mutation c.4837A > G exon 16 (25 patients), frameshift mutation c.5266dupC exon 20 (1 patient) and intronic mutation c.5277+48_5277+59dup (1 patient). Of the 53 patients studied, 26 were carriers of mutations, and only one patient presented the Ashkenazi founder mutation c.5266dupC; a mutation already identified in genetic studies in the Brazilian population. The mutation c.4837A>G occurred in 25 patients ruling out the possibility of being deleterious. Two of the mutations reported in this study do not yet have data on their molecular mechanisms in the literature, and thus demand further study in order to obtain a better understanding of the role of these mutations in the *BRCA1* gene in the Brazilian population, as well as for the rest of world.

Key words: Breast cancer, hereditary cancer syndrome, *BRCA1*.

1. Introduction

Breast cancer is probably the most frequent type of cancer and the second largest cause of cancer death among women [1]. In 2018, there was an increase in this cancer that corresponded to 24.2% of all new cancers diagnosed in women. It is estimated that, from 2018 to 2040, there will be an increase in the incidence of breast cancer of 46.3%, which will raise the number of deaths from 7.9 million to 13 million [2, 3].

According to the Brazilian National Cancer Institute, it is estimated that in Brazil 59,700 new cases of cancers are solely breast cancer, which corresponds to 29.5%. With regard to the incidence of the disease in the northern region of Brazil, breast cancer is the second most frequent among women, the first being cervical cancer. For Brazil, it is estimated that there

will be 66,280 new cases of breast cancer for each year of the 2020-2022 triennium. This figure corresponds to an estimated risk of 61.61 new cases per 100 thousand women. More specifically, for the state of Amazonas, the estimated occurrence rate for 2020 is 450 new cases of breast cancer for every 100 thousand women [4].

There are many risk factors that predispose people to breast cancer. Among them, some biological risk factors have been well established and involve age, characteristics of the woman's reproductive life (time of exposure to estrogen), breast tissue density, family history and presence of genetic changes. In relation to the risk factors that concern the lifestyle habits, the most common are nutrition, sedentary lifestyle, drinking and smoking, as well as exposure to ionizing radiation in younger women [5, 6].

Genetic factors also play a decisive role in some cases of breast cancer. Cancer-linked genes are

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categorized into three main types: cell proliferation inhibitors (tumor suppressors), proliferation activators (oncogenes), and those that repair DNA errors [5]. At least 10% of breast cancers occur in individuals with germinal mutations in high-penetrance genes. One of the first predisposing genes for breast cancer to be mapped was *BRCA1* from binding analyses involving families with numerous cases of breast cancer [7]. Recently, estimates have assessed that women who inherit a *BRCA1* mutation have a 72% chance of developing breast cancer and a 44% chance of developing ovarian cancer [8]. Mutations in the *BRCA1* gene may also predispose people to the development of other cancers, such as endometrial, pancreatic, colorectal, gastric and skin cancer [9, 10]. Genetic testing is therefore essential to identify individuals at risk in order to ensure an early diagnosis and reduce the risk of cancer, or at least the need for prophylactic surgeries (mastectomy and/or oophorectomy) [11].

Thus, it is necessary to find and characterize frequent mutations in each population. Currently, genetic testing is offered in several centers in North America, Europe, Australia and Israel, but is generally not available in South America. The performance of genetic testing is gaining greater worldwide acceptance in view of the number of preventive options that can be offered to women who have the mutation and because of the development of new and individualized therapies for cancer [12].

The most commonly used approach to finding individuals at high risk of being carriers of deleterious mutations that predispose to cancer is to analyze selected individuals based on the number and degree of kinship and family with cancer, age of diagnosis of breast tumors in individuals and the existence of other tumors [13]. In Brazil the panorama of frequency and prevalence of deleterious germ mutations in the population is not clear, but in recent years studies have been being conducted and published, which demonstrates an interest in modifying this scenario of lack of knowledge in Brazil [14, 15].

Although genetic studies in Brazil are increasing, there are no molecular research studies regarding the population of the state of Amazonas that would demonstrate the genetic cause of cancers treated in the state. The identification and characterization of these mutations in the Amazonian population are crucial for better treatment and prevention. Thus, the objective of this study was to characterize a group of patients with breast cancer treated at the Fundação Centro de Controle de Oncologia do Estado do Amazonas (FCECON) in regards to risk factors for hereditary breast cancer.

2. Materials and Methods

2.1 Samples and Ethical Aspects

A cross-sectional, descriptive study was conducted in the period from November 2014 to December 2018, 53 patients participated in the study who were selected after consultation in the outpatient clinic of the Fundação Centro de Controle de Oncologia do Estado do Amazonas (FCECON). The cohort was composed of 51 women and 2 men, from 30 to 71 years of age. All were diagnosed with breast cancer and had a family history and/or diagnosis at a young age (< 40 years of age), proven by clinical and histopathological examination at any stage of tumor development or medical referral report. The study was approved by the Research Ethics Committee of the Universidade do Estado do Amazonas, under approval number: 510.432. Each participating patient signed the informed consent form. After signing, a blood sample was obtained from each patient and this was labeled and sent to the Human Genetics Laboratory at the Universidade do Estado do Amazonas.

2.2 Extraction, Amplification and Sequencing

Genomic DNA was extracted from peripheral blood using the 2% CTAB standardized protocol by Doyle and Doyle [16]. The qualitative verification of the extracted DNA was performed by electrophoresis in 1% agarose gel. Polymerase chain reaction was

Table 1 Initiator oligonucleotide sequences for *BRCA1* gene exons.

Primers*	Sequence	Size/pb	AT**
Exon2	F: 5'GAAGTTGTCATTTTATAAACCTTT3' R: 5'TGTCTTTTCTTCCCTAGTATGT3'	250	60 °C
Exon3	F: 5'TCCTGACAGAGCAGACATTTA3' R: 5'TTGGATTTTCGTTCTCACTTA3'	340	60 °C
Exon8	F: 5'AAGCACAGAACTGGCCAACAA 3' R: 5'CACTTCCCAAAGCTGCCTAC 3'	274	60 °C
Exon9	F: 5'TGCCACAGGTAGATGCTCAGT 3' R: 5'CACATACATCCCTGAACCTAAA 3'	292	58 °C
Exon 13	F: 5'AATGGAAAGCTTCTCAAAGTA 3' R: 5'ATGTTGGAGCTAGGTCCTTAC 3'	322	62 °C
Exon 16	F: 5'AAAACCTCTTCCAGAATGTTGT3' R: 5'AATTCTTAACAGAGACCAAGAAC3'	452	56 °C
Exon 19	F: 5'CTGTCATTCTTCCTGGTGCTC3' R: 5'CATTGTAAAGGAAAGTGGTGTC3'	250	60 °C
Exon 20	F: 5'ATATGACGTGTCTGCTCCAC3' R: 5'GGGAATCCAAATTACACAGC3'	322	60 °C

*Primers: according to Dufloth et al., 2005. AT**: Annealing temperature.

performed to amplify eight exons of interest of the *BRCA1* gene of each patient (Table 1). The reaction profile was as follows: buffer 1X; magnesium chloride (MgCl₂) (2 mM); DNTPs (0.2 mM); primer F (0.5 µM) and primer R (0.5 µM); Taq DNA polymerase (1.25 U) (DNA Express Biotechnology®). PCR cycling was performed with initial denaturation for 5 min at 94 °C, followed by 40 cycles of 35 s at 94 °C, 40 s at 58-62 °C, an extension of 40s at 72 °C and a final extension for 10 minutes at 72 °C. PCR fragments were verified by electrophoresis in 1% agarose gel, cleaned with 20% polyethylene glycol (USB, Cleveland, USA). Bidirectional sequencing was performed with ABI BigDye™ Terminator v.3.1 sequencing reaction kit and an ABI 3130XL DNA analyzer (Applied Biosystems, Foster City, USA), following the manufacturer's instructions.

2.3 Data Analysis

The generated sequences were checked, edited and analyzed with the help of the Bioedit program, then were compared with the reference sequence *BRCA1*: NM_007294.3. For the annotation of the variants, the guidelines of the nomenclature of the Human Genome Variation Society (HGVS) (<http://varnomen.hgvs.org/>) were used. For the biological significance database of

all the reported variants we used ClinVar databases (www.ncbi.nlm.nih.gov/clinvar/), Varsome (<https://varsome.com/>), and BRCA Exchange (<http://brcaexchange.org/>) was also consulted.

3. Results

A total of 53 patients were tested for *BRCA1* mutations using direct sequencing of eight exons. The mean age of the patients was 50.4 years of age (ranging from 30 to 71 years of age). Family medical histories were available for 25 patients (47.16%). Among them, 92% had an immediate family member diagnosed with breast or ovarian cancer. Sequence analysis revealed four mutations (Table 2). Of the total number of patients analyzed in the present study, 26 presented mutations. Of these, 16 patients presented two concurrent missense mutations in exons 13 and 16. Nine patients had one mutation in one of the exons 13, 16 or 20, and one patient had three mutations simultaneously in exons 13, 16 and intron 20.

3.1 Characterization of Mutations

The missense mutation located in exon 13 is described as c.4304A>G and is characterized by an exchange of T→C (thymine for cytosine). This leads to the exchange of the amino acid ASP → GLY

Table 2 Alterations found in the *BRCA1* gene.

Location	HGVS*	Base exchanged	Amino acidexchanged	Typeofmutation	Significance	No. Of affected patients
Exon 13	c.4304A>G	T→C	Asp→Gly	Missense	Uncertainmeaning	16
Exon16	c.4837A>G	A→G	Ser→Gly	Missense	Benign	25
Exon20	c.5266dupC	insC	Stop codon	Frameshift	Pathogenic	1
Intron 20	c.5277+48_5277+59dup	dupGTATTCCACTCC		Intronic	Probablybenign	1

*Human Genome Variation Society.

(aspartate for a glycine), and this mutation was found in 16 patients (30.18%). As for the pathogenicity of this mutation, there is a disparity between the ClinVar, BRCA Exchange and Varsome databases, since the first and the second show it as a variation of uncertain significance (VUS), while the third assumes it to be probably benign.

Another missense mutation found and described as c.4837A>G is localized in exon 16 and is characterized by an exchange of A→G (adenine for guanine). This leads to the exchange of the amino acid SER→GLY (serine for a glycine), and this mutation was present in 25 patients (47.16%). The consulted databases, ClinVar, Varsome and BRCA Exchange, all classify this mutation as benign.

The frameshift change located in exon 20 is known as 5382insC and is described as c.5266dupC. It is characterized by the insertion of the nucleotide C and leads to a reading error from this point and the appearance of a stop codon at position 1829aa. This change was found in one patient who was diagnosed at the age of 63. The databases consulted, ClinVar, Varsome and BRCA Exchange, all classify this mutation as pathogenic.

The intronic mutation located in the 20 intron is described as c.5277+48_5277+59dup, and consists of the insertion of 12 base pairs in this intron (GTATTCCATCC). This was detected in only one patient (1.88%), who also presented missense mutations of exons 13 and 16. In the databases consulted, ClinVar and BRCA Exchange, this intronic mutation is classified as probably benign, whereas the Varsome database shows the classification as VUS.

Although the ClinVar database classifies it as a probably benign variant, there are no references to functional tests for such a classification. In the literature, to date, no citations about this mutation have been found.

4. Discussion

In Brazil, studies and data on mutations related to hereditary breast cancer are still scarce and are concentrated mainly in the south and southeastern regions of the country. Knowledge regarding mutations in germ lines of Brazilian patients with hereditary breast and ovarian cancer (HBOC) is limited. Few studies have carried out a large study on mutation in BRCA [14, 17-21]. Most studies focus on specific mutations or only a few regions of *BRCA1* and/or *BRCA2* [15, 22]. In the state of Amazonas, little is known about the genetic profile of patients diagnosed with early breast cancer and/or those with cases in the family. This study is the first to use the sequencing technique to track mutations in the *BRCA1* gene in the state population.

There are many factors that make it possible to develop breast or ovarian cancer. However, the largest and most prevalent factor is that of biological inheritance. The molecular diagnosis of hereditary breast cancer is complex and requires the analysis of the entire sequence of the *BRCA1* gene, and the differentiation of high or low risk variants is an important point for the discovery of clinical significance, as well as for a conclusive result of the genetic test [13, 23]. There are several clinical guidelines for patients with *BRCA* gene mutation,

including surgeries for risk reduction, such as total mastectomy and salpingo-oophorectomy [24], chemoprevention [25] and intensive surveillance with annual magnetic resonance imaging of the breast image [26].

The most common type of mutation found in the present study was the missense mutation observed in the two exons 13 and 16. The missense c.4304A>G mutation located in exon 13 classified as being of uncertain significance was present in 30.2% (16) of the patients. So far, there are no studies that demonstrate its frequency in the world or specifically in the Brazilian population and nor about its pathogenicity. Campilloa et al. [27], in a study with 111 women with HBOC from Spain, found two patients who presented this mutation, however, so far this is the only record.

Regarding the variant reported here, which was classified as having uncertain clinical significance (c.4304A>G), we observed a lack of consensus on its biological/clinical relationship among the different databases. There are examples, as reported by Fernandes et al. [18], where *BRCA2* variants c.4585G > A and c.6347A > G are considered pathogenic by the Human Gene Mutation Database/HGMD, without clinical significance by BIC and benign by ClinVar. These discrepancies between the databases, although “justified” by the low prevalence of most of these variants, make the clinical work and subsequent management of patients and their families very difficult. According to Miller-Samuel et al [28], the VUS can create additional confusion and anxiety for the patient and family, and increased uncertainty for the doctor in charge of medical management recommendations.

We know that Brazil is a country with an ethnically diverse population, and it has suffered the influence of many peoples, such as Europeans, Indians and Africans, and the mutation of c.4837A > G has already been described in two of these populations (European and African). Not surprisingly, the

missense change c.4837A > G located in exon 16, and classified as benign, was present in 47.2% (25) of the patients in this study. A study in Central Sudan by Biunno et al. [29] identified this mutation in 21/59 patients; a frequency that is similar to the one in this study, and one which suggests that this mutation may have entered the population due to the influence of the African populations. In Asian countries, such as Iran, a study confirmed the presence of this mutation in 50/88 women (7 with family history of cancer and 43 with sporadic cancer) [30]. Keshavarzi et al. [31] investigated all exons and introns of *BRCA1* in 85 women and reported that this mutation had a frequency of 21%.

In addition to these, Dombernowsky et al. [32], in a study of 419 women diagnosed with breast and/or ovarian cancer, evaluated the risk for these cancers associated with nine missense polymorphisms among them c.4837A > G, and found a frequency of 21% in the studied population. They also detected that heterozygosity or homozygosity of any combination of two of the nine polymorphisms was not associated with increased risk of breast and/or ovarian cancer. Yang et al. [33] performed a meta-analysis on this variation present in the literature and corroborated what has already been studied, indicating that this polymorphism may not be related to the risk of breast cancer, however they stressed that other studies should be conducted to confirm this result.

As mentioned previously, the Brazilian population has significant ethnic heterogeneity due to colonization by several peoples, which makes it difficult to find specific founding mutations. Nevertheless, among the mutations found in this study, one is the founder c.5266dupC. Da-Costa et al. [34] demonstrated through a haplotype profile study that the Ashkenazi mutation c.5266dupC originates in Eastern/Central Europe. This shows a significant contribution to the Brazilian population by the people that are native to these regions. Currently, the c.5266dupC mutation, located in exon 20, is the

second most frequent mutation worldwide described in the *BRCA* gene [14, 15, 35, 36]. However, despite its high frequency, it was detected in only one patient in this study.

An ancestry study was conducted for the c.5266dupC mutation, which involved 33 women, and 21 of these were breast cancer patients from the states of Rio de Janeiro, São Paulo and Porto Alegre. All the women with cancer presented this mutation and had mostly European ancestry, thus reinforcing the founder effect of this mutation in the Brazilian population [21]. This was corroborated by Kehdy et al. [37], who reported a European component that exceeds 70% in the south and southeast of Brazil, and which confirms European origin of the mutation c.5266dupC in those places where the mutation is found more frequent today. Some studies report an even higher European ancestry of 94.4% [18]. The frequency of this mutation is especially relevant among patients in the south and southeastern regions of Brazil, where the contribution of European ancestry is higher [21]. This justifies the low prevalence of this mutation in the north of the country.

The intronic mutation described as c.5277+48_5277+59dup and located in intron 20 was found in only one patient. Regarding this variant reported in this study, so far studies have not been found demonstrating its frequency in the world and Brazilian population, nor about its pathogenicity. It was classified as probably benign, due to the inserted bases being far from the splicing site. In general, intronic variants can affect alternative splicing mechanisms and gene transcription when localized in consensus sequences or accentuating/inhibitory sequences of these mechanisms [13].

It is known that advances in molecular techniques have helped to map mutations present in patients with hereditary breast cancer, but little is known about patients who present mutations in different exons of the same gene. In this study, we found patients who had more than one mutation in the

BRCA1 gene and one patient had three mutations in three different exons of *BRCA1*. A study conducted by Tung et al. [38], using a gene panel for breast cancer, revealed two patients who had 2 mutations in the CHEK2 gene, which is considered a high penetrance gene. This highlights the importance of studying the genetics of cancer so that patients with multiple mutations can receive appropriate treatment and receive the most accurate diagnosis.

This study found two mutations for which the molecular mechanisms are still unknown, which makes it necessary to carry out further studies in order to obtain a better understanding of the consequence of gene mutation in the Brazilian and world population. However, it is important to refine the knowledge of the Brazilian mutational pattern for high penetration genes for breast cancer, such as *BRCA1*, based on efficient genetic counseling and good use of molecular techniques, which would facilitate the follow-up and treatment of patients with this disease and monitoring their families.

It is important that genetic testing becomes affordable for this large number of Brazilian women diagnosed with breast cancer, since 70%-80% of the population depends on the public health system in Brazil, and this system does not provide genetic testing. The screening of mutations already established for *BRCA* can serve as the first strategic approach to genetic testing, not only in Brazil, but also in countries/health services where financial resources are limited and where the public health system or private plans do not cover molecular analysis. Due to the lack of genetic testing, the mutational profile of *BRCA* remains largely unknown. Systematic testing of individuals at risk provides knowledge of the genetic profile of a population and may allow the identification of multiple recurrent diseases and/or founder mutations that, in turn, would support the use of mutation panels as a first-line screening tool. If negative results are found through this initial screening, the analysis of all

coding sequences of *BRCA1* and *BRCA2* is imperative.

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