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Genetic polymorphism of calcium-sensing receptor in women with breast cancer

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Abstract

Breast cancer is a disease of unknown etiology, whose major risk factors are genetic alterations. Polymorphism of the calcium-sensing receptor (CaSR) has been a focus of some recent studies, due to a probable association with breast cancer risk and tumor aggressiveness. A relationship between polymorphic rs17251221 variant of the CaSR gene, and allele G (considered a gain-of-function mutation) and breast cancer risk has been stressed, despite the paucity of studies found in the literature. The present study involved 137 women (69 women with breast cancer—case; and 68 controls without breast cancer) who had 3 ml of peripheral blood drawn for DNA study. Genomic DNA was extracted from leukocytes by genotyping technique with real-time polymerase chain reaction. The AG genotype (rs17251221) was present in 13 women (18.84%) from the case group and in 8 (11.76%) women from the control group ($p = 0.3434$), while the GG genotype (rs17251221) did not occur in any group. In contrast, no statistically significant difference was observed between the AG genotype of variant rs17251221 in premenopausal case and control women ($p = 0.71$). There was also no statistically significant difference between postmenopausal case and control patients ($p = 0.6851$). In the current study, CaSR gene polymorphism of SNP variant rs17251221 did not show any statistically significant association with breast cancer, in both premenopausal and postmenopausal women.

Keywords Breast cancer · Calcium · CaSR polymorphism · Menopausal status · qPCR

Introduction

Breast cancer is the most common malignancy that affects women worldwide [1]. A 26% increase in the incidence of new breast cancer cases has been projected for the year 2020, mostly in developed countries [2]. In Brazil, which is a developing country, breast cancer is the disease with the highest incidence in the female population followed by non-melanoma skin cancer and the annual occurrence of breast cancer is rising and progressive, with 57,960 new cases and 14,206 deaths estimated for the year 2017 [3]. Breast cancer is a multifactorial disease of unknown etiology, and genetic alteration is the major risk factor for the disease [4]. Likewise, genetic mutations, such as breast cancer (BRCA) genes 1 and 2, may increase the lifetime risk of developing hereditary breast and ovarian cancers [5].

Nevertheless, the calcium-sensing receptor gene (CaSR) has also been associated with breast cancer risk [6, 7]. The CaSR gene is located on the long arm of

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chromosome 3, and it is a ubiquitously expressed class C G-protein coupled receptor (GPCR). Furthermore, the CaSR gene is the master regulator of calcium homeostasis which is highly expressed in the parathyroid, thyroid and kidneys [8, 9]. However, the CaSR gene is also expressed in a variety of tissues unrelated to calcium homeostasis, such as the skin, brain and breast [10, 11], where it regulates cell functions such as differentiation, proliferation, apoptosis and gene expression [12]. Single-nucleotide polymorphism (SNP) is the most prevalent form of genetic variation in the human genoma [13]. The polymorphic SNP rs17251221 variant of the CASR gene, and allele G (considered a gain-of-function mutation), has been associated with a higher risk of breast cancer [6]. Nevertheless, there are few studies published in the literature on the subject and thus we were motivated to design the current study in Brazilian women.

Materials and methods

Patients and blood samples

This is a controlled cross-sectional study, involving patients managed in the Outpatient Clinic of Breast Disorders of the Getúlio Vargas (HGV) Hospital and Molecular Biology Laboratory of the Natan Portela Hospital, Federal University of Piauí, Brazil, from May 2010 to September 2015. The study included 137 women, divided into case (with breast cancer, $N = 68$) and control (without breast cancer, $N = 69$) groups. Included in this study were patients with histologically confirmed breast cancer and healthy women (controls), evaluated by physical examination and imaging tests that were negative for malignancy. Excluded from the study were women older than 80 and patients suffering from liver, metabolic, cardiovascular, or kidney disease or those reporting other types of malignancies. Three milliliters of blood was drawn by a specialized technician using a disposable syringe and needle after the patient had undergone medical consultation. Whole blood was stored in proper vials containing anticoagulant (EDTA), stored in a freezer, at − 20 °C. All women in the study were instructed to avoid using anti-inflammatory agents within 72 h of blood sample collection.

DNA extraction

For DNA extraction of sample leukocytes, the PureLink Genomic® DNA Mini Kit (Life Technologies) was used, following the manufacturer's instructions.

Genotyping

After isolation, DNA concentration was determined by Nanodrop 2000 spectrophotometry (Thermo Fisher Scientific, Waltham, MA, USA). Genotyping was performed by real-time polymerase chain reaction (RT-PCR). The RT-PCR technique allows the detection of fluorescence intensity due to amplification of target DNA sequence during each cycle, with an elevated sensitivity and specificity. It enables a comparative analysis of gene expression among samples at the starting point of the exponential amplification phase, in which there is no saturation of amplification. As Taq polymerase enzyme replicates DNA in each cycle of PCR, a fluorophore that emits fluorescent light is released. Quantification of fluorescence emission indicates the exact number of DNA copies initially present. Absolute DNA quantification in a sample is performed with the use of a standard curve, obtained by amplification of known quantities of the same DNA. Genotyping assays of SNP contain a probe labeled with VIC® dye and a probe labeled with FAM™ dye. TaqMan® probes incorporate MGB technology, where the VIC® probe detects allele 1 and the FAM™ probe detects allele 2. The reactions were conducted in final volumes of 20 µl per patient, containing: 10 µl TaqMan® Genotyping Master Mix; 0.5 µl of TaqMan® probe customized for genotyping of SNPs of the CaSR human gene (SNP ID rs17251221. Cod. C__32771445_10 Context sequence VIC/FAM: TATAAATAAATGTTTGTCTAAAAA T[A/G]AAGTTAATACAGATATCAATTGTTA) (Table 1); 5.5 µl of deionized DNA/RNA-free water; and 4 µl of DNA sample per patient; these volumes were distributed in 96-well reaction plates (MicroAmp® Fast Optical 96-Well Reaction Plate), 0.1 ml (Applied Biosystems, EUA) in duplicate. Amplification was performed by using Fast Real-Time PCR System 7500 with SDS 2.2 software incorporated for SNP genotyping (Applied Biosystems, EUA), in the following steps: 1-Pre-PCR, with a duration of 1 min at 60 °C; 2-Pre-incubation of the reaction mixture at 95 °C during 10 min; 3-thermocycling at 95 °C during 15 s and 60 °C for 60 s for 40 cycles; 4-Post-PCR, with a duration of 1 min at 60 °C. Fluorescence data were captured during 40 reaction cycles. Quality control of

Table 1 Identification of gene code and SNP used in TaqMan® assay. *Source*: Life Technologies

Gene variant CaSR	Sequence context VIC/FAM
rs17251221A>G	TATAAATAAATGTTTGTCTAAAAAT[A/G]AAGTTAATACAGATATCAATTGTTA

RT-PCR was assessed by random selection of 20% of the total samples for re-genotyping by an independent technician.

Statistical analysis

The Chi-square test was used to determine whether genotype distribution conformed to Hardy–Weinberg equilibrium. Genotype frequency was compared between women with breast cancer and women without the disease from a control group, using Fisher's exact test. The odds ratio (OR) and 95% confidence interval (CI) were calculated using Fisher's exact test due to low frequencies in lines. Statistical significance was set at $p < 0.05$.

Results

This study included 137 women (69 cases, 68 controls). Mean patient age and standard deviation were 49.0 ± 11.2 years for cases and 45.4 ± 12.7 for controls. Genotype frequencies of variant rs17251221 of CaSR conformed to Hardy–Weinberg equilibrium. AG genotype (rs17251221) occurred in 13 (18.84%) case women and in 8 (11.76%) controls ($p = 0.3434$), while the GG genotype (rs17251221) did not occur in any of the groups (Table 2).

After stratification according to menopausal status, no statistically significant difference was observed between AG genotype of variant rs17251221 in 8 (20.51) premenopausal women from the case group and 6 (13.04%) women from the control group ($p = 0.67$). No difference was found in women of postmenopausal status, five cases (16.66%) and 2 controls (9.09%) ($p = 0.6851$). In contrast, genotype GG did not occur in any of the groups (Table 3).

Discussion

The distribution of genetic variants differs between populations of diverse ethnic and geographic backgrounds. Genetic variants in the Brazilian population do not commonly show a consistent pattern of distribution due to widespread miscegenation, since it differs from other countries where populations are predominantly Caucasian, African or Asian [14, 15]. CaSR gene polymorphism has been related to increased calcium levels, reduced calcium-sensing receptor expression and a higher breast cancer risk and tumor aggressiveness [16]. Nevertheless, to the best of our knowledge, few studies in the literature have associated CaSR gene polymorphism, particularly its variant rs17251221, with breast cancer risk [7, 16, 17]. In addition, until now, there are no studies on

Table 2 Patient characteristics and genotyping of CaSR gene SNP rs17251221 in case and control patients

Characteristics	Case			Control
N	69			68
Mean age (SD)	49.0 (± 11.2)			45.4 (± 12.7)
Premenopausal	39			46
Postmenopausal	30			22
	Case (%)	Control (%)	p	OR (95% CI)
rs17251221				
AA	56 (81.16)	60 (88.24)	0.3434	0.5768
A/G	13 (18.84)	8 (11.76)	–	1
GG	–	–	–	–

There was no statistically significant difference of SNP genotype (rs17251221) between case and control groups ($p > 0.05$). The odds ratio (OR) was not significantly higher between groups

Table 3 Genotyping of CaSR gene SNP rs17251221 in premenopausal and postmenopausal patients

	Premenopausal				Postmenopausal			
	Case	Control	p	OR (95% CI)	Case	Control	p	OR (95% CI)
rs17251221								
AA	31	39	0.3977	0.59988	25	21	0.6851	0.4826
A/G	8	6	0.67	[0.15; 2.21]	5	2	–	1
GG	–	–	–	–	–	–	–	–

There was no statistically significant difference between SNP genotype rs17251221 in the case group and premenopausal control group ($p > 0.05$), as well as in the postmenopausal group ($p > 0.05$). There was no significantly higher odds ratio (OR)

the subject in the Brazilian population. In the present study, no statistically significant difference was observed between genotypes of case and control groups. There was also no difference between the above-mentioned groups regarding premenopausal and postmenopausal status for variant rs17251221.

The results of this study did not show any association between variant rs17251221 SNP and breast cancer risk in cases and controls. However, in a study including 217 female breast cancer women and 231 controls, Li et al. [7] found that AG and GG genotypes were associated with lower mRNA and protein levels of CaSR, in comparison with the AA genotype in breast cancer tissues. Therefore, those authors concluded that rs17251221 SNP is a risk factor associated with breast cancer susceptibility, as well as a prognostic indicator.

Yao et al. [17] conducted a research study of the African American Breast Cancer Epidemiology and Risk (AMBER) consortium that was composed of four studies involving 3663 breast cancer cases and 4687 controls. The aim of the study was to evaluate genetic variations in vitamin D-related pathways and breast cancer risk. Those authors found evidence of an association between vitamin D-related genetic variations and breast cancer risk, particularly tumor ER- breast cancer. The authors found that CaST may be related to tumor ER status, supporting the role of vitamin D or calcium modifying breast cancer phenotypes. In turn, in a case–control study including 199 breast cancer cases and 384 controls without breast cancer, Wang et al. [16] observed that reduced sensitivity of the CaSR to calcium due to inactivated polymorphisms at rs1801725 may predispose up to 20% of breast cancer to high circulating levels of calcium associated with larger and more aggressive tumors.

In the current study, there was no statistically significant association between CaSR gene polymorphism, variant rs17251221 and breast cancer risk, in both premenopausal and postmenopausal women. However, further studies with larger sample size are required in Brazilian women.

Compliance with ethical standards

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