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Associação dos polimorfismos de nucleotídeo único rs63751445 do gene *MSH2* e rs863224614 do gene *MSH6* com a suscetibilidade ao câncer de mama em amostras da região Nordeste do Brasil

Association of single nucleotide polymorphisms rs63751445 of the *MSH2* gene and rs863224614 of the *MSH6* gene with susceptibility to breast cancer in samples from Northeast Brazil

Asociación de polimorfismos de un solo nucleótido rs63751445 del gen *MSH2* y rs863224614 del gen *MSH6* con susceptibilidad al cáncer de mama en muestras del Noreste de Brasil

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Resumo

O câncer de mama (CM) é o câncer de maior impacto epidemiológico na população feminina do mundo inteiro. A doença apresenta etiologia multifatorial, com implicações genéticas que não estão totalmente elucidadas. Neste contexto, alterações genéticas do mecanismo de reparo de mal pareamento são destacáveis pela relação potencial com o CM, em especial os polimorfismo de nucleotídeo único (SNPs), que são o tipo de variação genética mais comum. O objetivo deste estudo foi avaliar pela primeira vez a influência dos SNPs rs63751445 (A>G) do gene *MSH2* e rs863224614 (T>G) do gene *MSH6* para a susceptibilidade ao CM. Para isso, utilizaram-se 100 amostras obtidas por exame histopatológico de pacientes da

região Nordeste do Brasil. A metodologia utilizada foi o método Didesóxi Único Alelo Específico PCR (DSASP). A análise estatística foi feita pela comparação com a população controle (população em equilíbrio de Hardy-Weinberg) através dos testes Qui-quadrado de Pearson e exato de Fischer. Concluí-se que estes dois SNPs podem estar associados à suscetibilidade ao CM na população estudada.

Palavras-chave: Câncer de mama; Mecanismo de reparo de mal pareamento; Polimorfismo de ucleotídeo único; DSASP; Genotipagem.

Abstract

Breast cancer (BC) is the cancer with the greatest epidemiological impact on the female population worldwide. The disease has a multifactorial etiology, with genetic implications that are not fully understood. In this context, genetic changes in the mismatch repair mechanism are notable for their potential relationship with BC, especially the single nucleotide polymorphisms (SNPs), which are the most common type of genetic variation. The aim of this study was to evaluate for the first time the influence of the SNPs rs63751445 (A>G) of the *MSH2* gene and rs863224614 (T>G) of the *MSH6* gene for susceptibility to CM. For that, 100 samples obtained by histopathological examination of patients from the Northeast region of Brazil were used. The methodology used was the Didesoxy Single Allele Specific PCR (DSASP) method. Statistical analysis was performed by comparison with the control population (population in Hardy-Weinberg equilibrium) using Pearson's Chi-square and Fischer's exact tests. It was concluded that these two SNPs may be associated with susceptibility to BC in the studied population.

Keywords: Breast cancer; Mismatch repair mechanism; Single nucleotide polymorphism; DSASP; Genotyping.

Resumen

El cáncer de mama (CM) es el cáncer con mayor impacto epidemiológico en la población femenina a nivel mundial. La enfermedad tiene una etiología multifactorial, con implicaciones genéticas que no se comprenden completamente. En este contexto, los cambios genéticos en el mecanismo de reparación de desajustes son notables por su posible relación con lo CM, especialmente los polimorfismos de un solo nucleótido (SNP), que son el tipo más común de variación genética. El objetivo de este estudio fue evaluar por primera vez la influencia de los SNPs rs63751445 (A>G) del gen *MSH2* y rs863224614 (T>G) del gen *MSH6* para la susceptibilidad a CM. Para ello, se utilizaron 100 muestras obtenidas por examen

histopatológico de pacientes de la región Nordeste de Brasil. La metodología utilizada fue el método Didesoxy Single Allele Specific PCR (DSASP). El análisis estadístico se realizó por comparación con la población de control (población en equilibrio de Hardy-Weinberg) utilizando las pruebas de Chi-cuadrado de Pearson y exactas de Fischer. Se concluyó que estos dos SNP pueden estar asociados con la susceptibilidad a CM en la población estudiada.

Palabras clave: Cáncer de mama; Mecanismo de reparación de mal apareamiento; Polimorfismos de un solo nucleótido; DSASP; Genotipado.

1. Introduction

Breast cancer (BC) is the most common type of cancer in the female population worldwide. It is a multifactorial disease, with etiology often implicated in genetic factors (Nogueira et al., 2020). In this context, BC can be studied by addressing genetic changes that occur in proto-oncogenes, tumor suppressor genes and DNA repair genes, among which are mutations and single nucleotide polymorphism (SNP) (Sun et al., 2017).

SNPs are genetic changes to a single nucleotide. They are commonly influencing susceptibility to diseases and, therefore, are important targets for association studies involving multiple types of cancer (Tawfik & Spruit, 2018).

The mismatch repair mechanism (MMR) is conserved in prokaryotic cells, such as those of *Escherichia coli*, and in eukaryotic cells, such as those of *Homo sapiens*. The eukaryotic cell MMR starts when one of the following two protein complexes recognizes and interacts with the error site: MutSα (MSH2-MSH6) and MutSβ (MSH2-MSH3). Then, the recognition complex initiates a cascade of recruitment of other proteins involved in the repair, such as MLH1-PMS2, Exon 1, PCNA and RPA (Martín-López & Fishel, 2013).

The *MSH2* gene (MIM#609309) has a 2p21-p16 cytogenetic location and the *MSH6* gene (MIM#600678) has a 2p16.3 cytogenetic location. The proteins synthesized by the two genes interact to form the MMR primer complex, capable of recognizing the incorrect pairing between two bases or insertions/deletions (Silva et al., 2009).

MMR SNPs have been associated with the susceptibility to cancer development in multiple studies (Win et al., 2013; Santos et al, 2018), including BC (Kappil et al., 2016). However, many association studies still need to be done and, according to Pereira et al. (2018), the methodological approach to a scientific problem can be made through the hypothetical method.

In this context, the present work is based on applied research with quantitative methodology to test for the first time the hypothesis of association between SNPs rs63751445 (A>G) of the *MSH2* gene and rs863224614 (T>G) of the *MSH6* gene with BC susceptibility population in the Northeast region of Brazil.

2. Methodology

2.1 Sampling

109 samples were received from the Laboratory of Pathological Anatomy and Clinical Analysis UNILAB - João Pessoa, where the extraction of cancerous breast tissue was performed by histological technique and the subsequent paraffinization. However, only 100 samples were selected for this study according to the criteria of tissue quality, completeness of patient data and collection time of, at most, five years ago.

2.2 Ethical assessment

The study obtained a favorable opinion from the Research Ethics Committee of the Hospital-School of the Federal University of Paraiba – UFPB (Universitary Hospital Lauro Wanderley) under the code of Certificate of Presentation for Ethical Appreciation (CAAE) nº 08697219.7.0000.8069.

2.3 DNA extraction

The extraction was done at the Laboratory of Structural Molecular Biology and Oncogenetics (LBMEO) at UFPB through the modification of the method of Shi et al. (2002). In our study, the paraffined samples were cut and dewaxed heated xylol and then incubated in 20.0 µL of proteinase K at 10.0 mg/mL and 1.0 mL of extraction buffer (Tween 20 0.45%, Triton 1% X-100, 0.01 M Tris/HCl, 50 mM KCl, 5 mM MgCl 2, 0.45% NP-40). It was heated to 57ºC for 3 hours.

The next steps were: adding the phenol/chloroform/isoamyl alcohol mixture (25:24:1) followed by homogenization by gentle inversion for 5 minutes and centrifugation at 3000 rpm for 5 minutes. Then, the supernatant was purified with chloroform/isoamyl alcohol (24:1). The DNA was precipitated with chilled absolute ethanol over centrifugation at 10,000 rpm for

3 minutes, the supernatant was discarded. DNA was dehydrated at 60ºC and 200.0 mL of sterile Milli-Q water was used to resuspend.

2.4 Genotyping

This step was performed using the Didesoxy Single Allele Specific PCR (DSASP) method, whose fundamental principle is the allelic discrimination resulting from the incorporation of ddNTP in the position of the SNP of interest. This method has three steps: (I) asymmetric PCR – step of amplification of only one of the two strands of DNA according to the specificity determined by the primer; (II) hybridization – step of synthesis of a complementary strand that, according to the polymorphic variant present, can be interrupted by ddNTP; (III) melting curve - instrumental analysis capable of discriminating the strands corresponding to the two polymorphic variants (Lima et al., 2015).

2.5 Validation in silico

The design and analysis of the stability of the primers was made through bioinformational resources provided by National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/), Ensembl Genome Browser (https://www.ensembl.org/index.html) and Gene Runner version 6.5.52.

2.6 PCR conditions

The PCR mixture for the SNP rs63751445 (*MSH2*) was composed of 12.5 µL of ultrapure water, 3.0 µL of buffer, 1.0 µL of 25 nM $MgCl_2$(aq), 4.0 µL of the mixture composed of dNTP's and ddNTP, 1.5 µL of Primer F, 300 ng/µL of DNA and 1 U of Taq polymerase. The PCR steps for this SNP were: (I) pre-denaturation at 95ºC for 5 minutes; (II) cycle (40 times) at 95ºC for 20 seconds, 51ºC for 30 seconds and 72ºC for 20 seconds; and (III) final extension at 72ºC for 2 minutes.

The PCR mixture for the SNP rs863224614 (*MSH6*) was composed of 13.0 µL of ultrapure water, 3.0 µL of buffer, 2.0 µL of 25 nM $MgCl_2$(aq), 4.0 µL of the mixture composed of dNTP's and ddNTP, 1.0 µL of Primer F, 300 ng/µL of DNA and 1 U of Taq polymerase. The PCR steps for this SNP were: (I) pre-denaturation at 95ºC for 5 minutes; (II) cycle (80 times) at 95ºC for 20 seconds, 55ºC for 30 seconds and 72ºC for 20 seconds; and (III) final extension at 72ºC for 2 minutes.

2.7 Hybridization conditions

The hybridization of the PCR products was carried out by adding 1.5 mL of specific complementary sequence to the mixture and submitting to the following conditions: preheating from 25ºC to 95ºC for 1 minute, doubling to 45ºC for 5 minutes, and a gradual heating (1ºC per minute) up to a temperature of 95ºC for 5 minutes.

2.8 Melting Curve Analysis

In this step, the 7500 Fast Real-Time PCR System (Life Technologies - Carlsbad, CA) was used. The steps were: preheating from 25ºC to 95ºC for 1 minute, doubling to 45ºC for 5 minutes, and a gradual heating (1ºC per minute) to a temperature of 95ºC for 5 minutes.

2.9 Statistical analysis

The Hardy-Weinberg equilibrium equations were used to obtain the expected results from the results obtained experimentally. Statistical analysis was performed using Pearson's Chi-square and Fischer's Exact tests. Only $p < 0.001$ was considered statistically significant.

3. Results and Discussion

MMR is indispensable for replication fidelity and is preserved in prokaryotic and eukaryotic cells. This mechanism is performed in human cells by two classes of homologous proteins, MSH, which recognizes the site of mismatch, and MLH, which triggers the cascade of activation of proteins responsible for the repair itself (Martín-López & Fishel, 2013).

Genetic changes in MMR can direct the cell to carcinogenesis. In this context, SNPs stand out, which can often be associated with cancer regardless of whether coding and non-coding regions occur (Fagny et al., 2019). Our results suggest that neither SNPs rs63751445 (A>G) of the *MSH2* gene (Table 1) and rs863224614 (T>G) of the *MSH6* gene (Table 2) is associated with the age or anatomical location variable ($p > 0.001$).

Table 1 – Distribution of age and anatomical location data for the SNP rs63751445.

Variable	Total	Genotype AA	Genotype AG	Genotype GG	p value
Age (years)					
age < 50	36	32	0	4	0.7178
50 ≤ age ≤ 69	47	42	0	5	
age > 69	17	14	0	3	
Anatomical location					
Left breast	47	43	0	4	0.3684
Right breast	53	45	0	8	

Fonte: Own authorship (2020).

Table 2 – Distribution of age and anatomical location data for the SNP rs863224614.

Variable	Total	Genotype TT	Genotype TG	Genotype GG	p value
Age (years)					
age < 50	36	33	0	3	0.1743
50 ≤ age ≤ 69	47	40	0	7	
age > 69	17	12	0	5	
Anatomical location					
Left breast	47	39	0	8	0.7800
Right breast	53	46	0	7	

Fonte: Own authorship (2020).

We did not obtain an association between the SNPs rs63751445 (A>G) of the *MSH2* gene and rs863224614 (T>G) of the *MSH6* gene with the age variable. However, it is possible to make important observations in Tables 1 and 2. Regarding the age of diagnosis, our study showed the concentration of cases in the age group over 35 years, which is corroborated by reports by Mattos et al. (2020), Kamińska et al. (2015) e Azevedo et al. (2017). In addition, the previous tables have a high incidence in the age group below 50 years (36% of cases), which may be related to the tendency of increasing BC in young women. This trend was observed in a study on the incidence of BC in American women in the period from 1935 to 2015 (Lima et al., 2020).

Regarding the variable anatomical location, it was observed in Tables 1 and 2 that there is no significant difference between the incidence in the breasts on both sides, that is, the

incidence rate in the left and right breasts was approximately equal. Other authors have already described approximate values of BC incidence in both breasts in studies with thousands of cases (Pinheiro et al., 2013).

The results of this study are favorable to the hypothesis that both SNPs are associated with susceptibility to BC (Table 3). Comparing the genotypic frequencies of the studied population and the control population (population governed by the Hardy-Weinberg Equilibrium), p values < 0.001 were obtained for both SNPs studied, that is, the difference between the two populations is statistically significant.

Table 3 – Allelic and genotypic distribution of SNPs of the *MSH2* and *MSH6* genes in individuals with BC.

Gene/SNP	**Observed genotypic frequencies**	**Gene frequencies**	**Expected genotype frequencies**	***χ2***	**p value**
MSH2 rs63751445	AA: 88 (88%) AG: 0 (0%) GG: 12 (12%)	A: 0.88 (88%) G: 0.12 (12%)	AA: 77.44 (77.44%) AG: 21.12 (21.12%) GG: 1.44 (1.44%)	30.091	2.923 x 10^{-7}
MSH6 rs863224614	TT: 85 (85%) TG: 0 (0%) GG: 15 (15%)	T: 0.85 (85%) G: 0.15 (15%)	TT: 72.25 (72.25%) TG: 25.50 (25.50%) GG: 2.25 (2.25%)	35.958	1.556 x 10^{-8}

Fonte: Own authorship (2020).

Previous studies have already associated the occurrence of multiple SNPs with breast carcinogenesis (Rath et al., 2020). In this study, the SNPs rs63751445 (A>G) of the *MSH2* gene and rs863224614 (T>G) of the *MSH6* gene were associated with susceptibility to breast carcinogenesis in the population of the State of Paraíba, Brazil (p < 0.001). Li & Martin (2016) highlighted *MSH2* and *MSH6* among the MMR genes most commonly mutated in colorectal cancer pathology.

Regarding the SNPs of the *MSH2* gene, Li *et al.* (2012) and Sun *et al.* (2014) found an association with statistical significance between the G allele of SNP rs63749993 of the *MSH2* gene and susceptibility to esophageal cancer. In addition, the authors of this second study observed increased susceptibility in the simultaneous occurrence of the G allele of the SNP rs63749993 of the *MSH2* gene with the G allele of the SNP rs9876116 of the *MLH1* gene. In

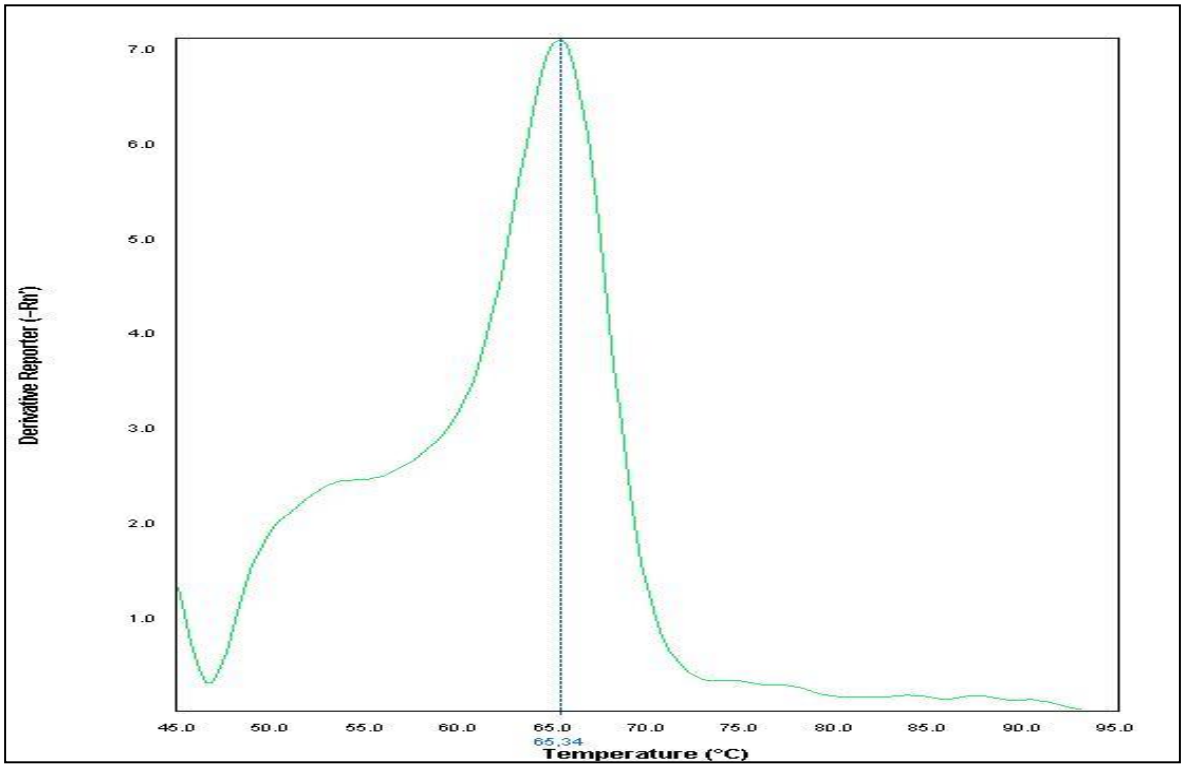
addition, Calixto et al. (2018) used the DSASP method to prove the association of SNPs rs565410865 (G>T) of the *MLH1* gene and rs560246973 (C>T) of the *MSH2* gene to susceptibility to the development of basal cell carcinoma in a population sample in the state of Paraíba - Brazil.

Corroborating the relevance of the SNPs of the *MSH6* gene to cancer, the study by Santos *et al.* (2019) highlighted the association between SNP rs1042821 of the *MSH6* gene and other SNPs of MMR genes at risk for thyroid cancer.

Therefore, multiple SNPs of the *MSH2* and *MSH6* genes have already been associated with different types of cancer. This demonstrates that the hypothesis of an association between the SNPs rs63751445 (A>G) of the *MSH2* gene and rs863224614 (T>G) of the *MSH6* gene is viable and the susceptibility to BC in the studied population.

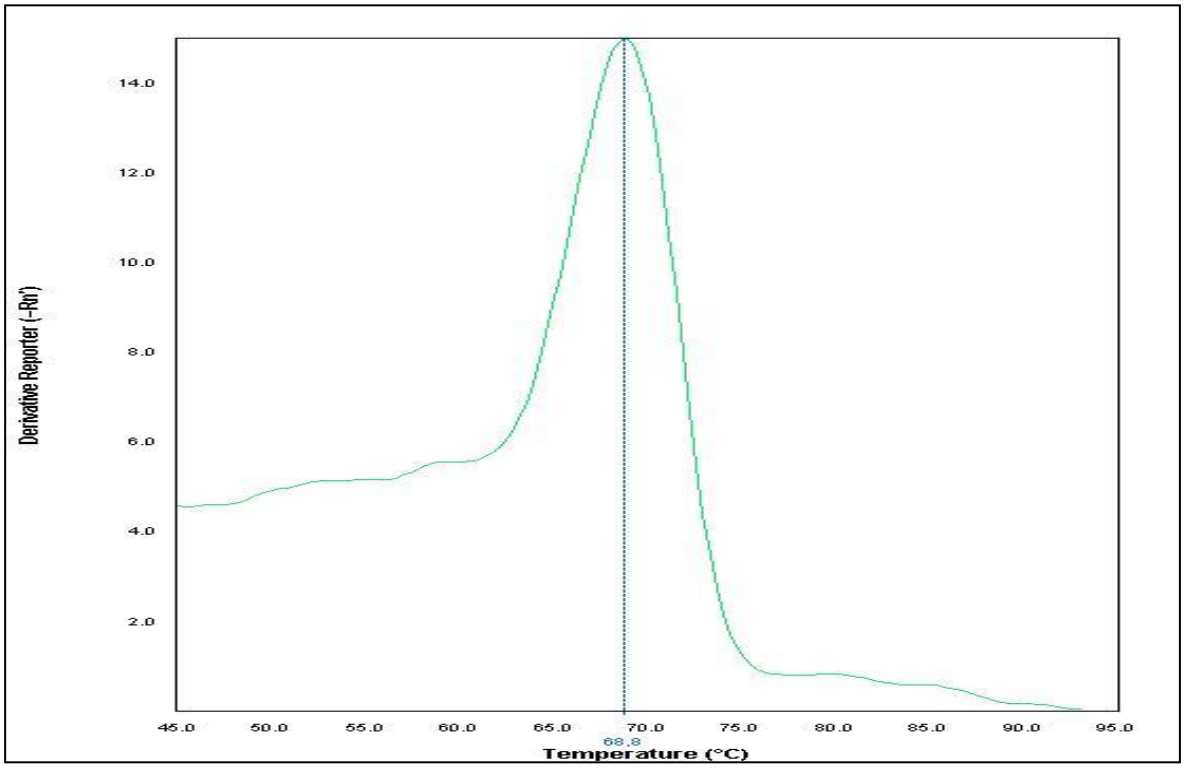
Figures 1, 2, 3 and 4 show that the melting curves obtained were representative of homozygotes, which can be evidenced by the presence of a single peak in these graphs. In this work, no melting curves representative of heterozygotes were found (curves with a double peak characteristic of the presence of the native allele and the polymorphic allele). The melting temperature for the A allele of the SNP rs63751445 (*MSH2*) varied between 65.34ºC and 66.98ºC (Figure 1). The melting temperature for the G allele of the SNP rs63751445 (*MSH2*) varied between 67.10ºC and 68.80ºC (Figure 2). The melting temperature for the T allele of the SNP rs863224614 (*MSH6*) varied between 58.83ºC and 60.65ºC (Figure 3). The melting temperature for the G allele of the SNP rs863224614 (*MSH6*) varied between 63.57ºC and 66.66ºC (Figure 4).

Figure 1 - Melting curves representative of the SNP rs63751445 allele A of the *MSH2* gene.



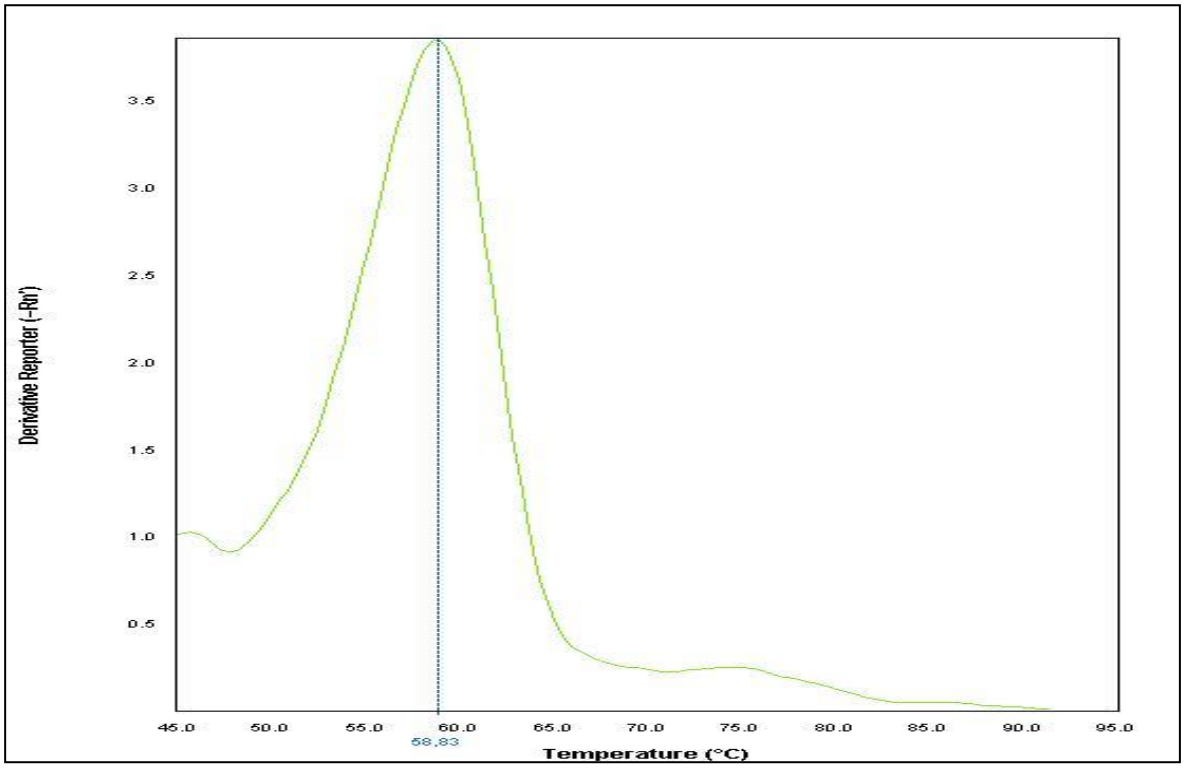
Fonte: 7500 Software v2.0.6 (Life Technologies – Carlsbad, CA).

Figure 2 - Melting curves representative of the SNP rs63751445 allele G of the *MSH2* gene.



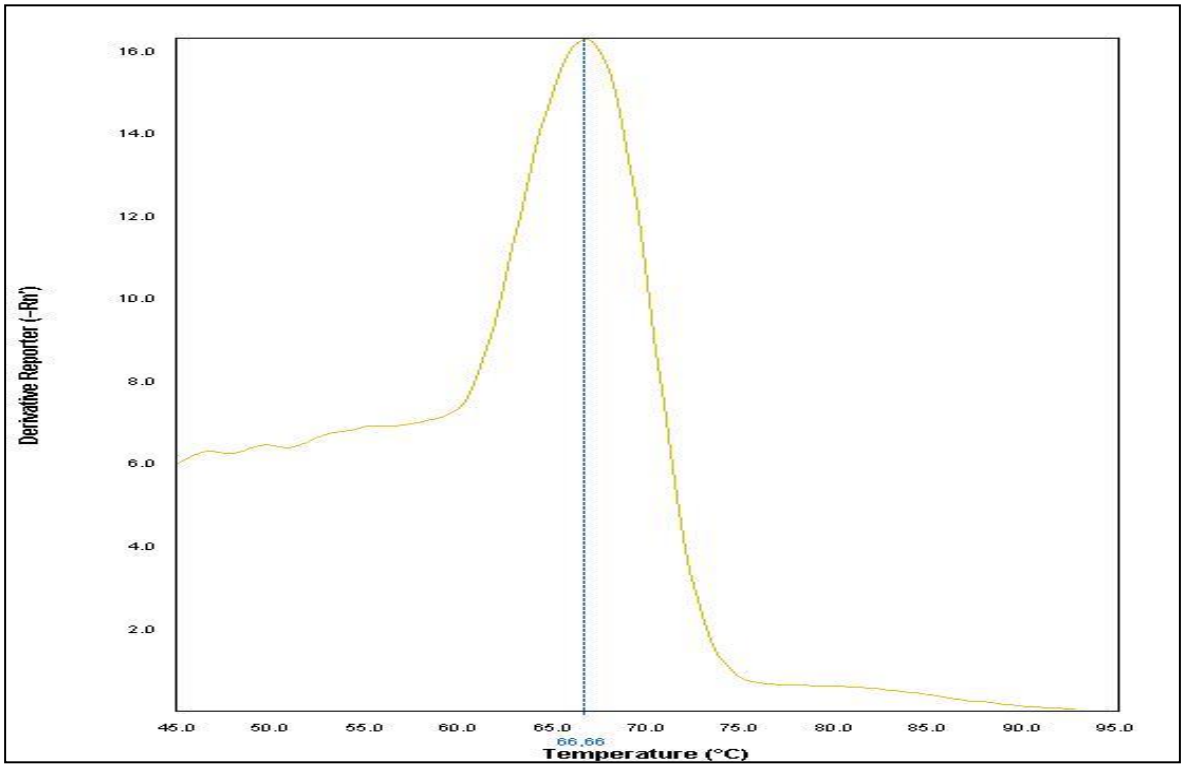
Fonte: 7500 Software v2.0.6 (Life Technologies – Carlsbad, CA).

Figure 3 - Melting curves representative of the SNP rs863224614 allele T of the *MSH6* gene.



Fonte: 7500 Software v2.0.6 (Life Technologies – Carlsbad, CA).

Figure 4 - Melting curves representative of the SNP rs863224614 allele G of the *MSH6* gene.



Fonte: 7500 Software v2.0.6 (Life Technologies – Carlsbad, CA).

Although melting curves representative of heterozygotes were not observed in this work (Figures 1, 2, 3 and 4), the PCR methodology described in this work was not designed to detect the phenomenon of loss of heterozygosity. We suggest that, to test this hypothesis, PCR methodologies based on the work of Farrand et al. (2002). We also emphasize that the number of cases studied in this work is insufficient to propose the SNPs rs63751445 (A>G) of the *MSH2* gene and rs863224614 (T>G) of the *MSH6* gene as molecular markers of BC.

4. Final Considerations

The results suggest that the SNPs rs63751445 (A>G) of the *MSH2* gene and rs863224614 (T>G) of the *MSH6* gene are associated with susceptibility to BC in the studied population.

Finally, we clarify that we do not aim to conclude, based solely on this work, that: (I) the phenomenon of loss of heterozygosity has occurred in the population studied; or (II) the SNPs considered to be molecular markers of BC. However, both hypotheses can be tested in further studies. The first hypothesis must be tested by methodologies that are different from the PCR methodology of this work, such as, for example, an adaptation of the methodology

by Farrand et al. (2002). The second hypothesis should be tested in a study with a greater number of cases.

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