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ORIGINAL ARTICLE

Single-Nucleotide Polymorphisms of the MSH2 and MLH1 Genes, Potential Molecular Markers for Susceptibility to the Development of Basal Cell Carcinoma in the Brazilian Population

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Abstract Basal cell carcinoma - BCC is considered a multifactorial neoplasm involving genetic, epigenetic and environmental factors. Where UVB radiation is considered the main physical agent involved in BCC carcinogenesis. The Brazil and state of Paraíba are exposed to high levels of UVB rays. The mismatch repair - MMR is important DNA repair mechanisms to maintain replication fidelity. Therefore, single nucleotide polymorphisms (SNPs) in genes encoding proteins involved in MMR may be potential molecular markers of susceptibility to BCC. The objective of this study was to evaluate and describe for the first time the SNPs rs560246973, rs2303425 and rs565410865 and risk of developing BCC. The present study analyzed 100 samples of paraffin-embedded tissue from patients with histopathological diagnosis of BCC and 100 control samples. The results were obtained by genotyping method, Dideoxy Unique Allele Specific – PCR (DSASP). The SNPs rs2303425 were not associated with Basal Cell Carcinoma. However, the SNPs rs560246973 and rs565410865 was shown to be associated with the development of BCC when compared to control samples ($P < 0.0001$). The SNPs rs565410865 was also statistical significance between the genotypes of and the age group ($p = 0.0027$) and tumor location ($p = 0,0191$). The result suggests that SNPs rs2303425 and rs565410865 are associated with susceptibility to the development of BCC in the Brazilian population and may be considered as potential molecular markers for BCC.

Keywords Basal cell carcinoma · Mismatch repair · Single nucleotide polymorphism · DSASP · Genotyping · Molecular markers

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Introduction

Basal cell carcinoma - BCC is considered a multifactorial neoplasm involving genetic, epigenetic and environmental factors [1–3]. Where UVB radiation is considered the main physical agent involved in BCC carcinogenesis [4].

Table 1 Primer and complementary sequence for genotyping the SNP *rs565410865* of the MLH1 gene and the SNPs *rs560246973* and *rs2303425* of the MSH2 gene

Gene	SNPs	Primers/ Complementary sequence
MLH1	*rs565410865*	5′-CTTTGCTTACTTGGTGTCTCTAGTTCTGG-3′
		5′-CAGGMACCAGAACTAGAGACACCAAGTAAGCAAAG-3’
MSH2	*rs560246973*	5’-AATCCATTATGTTTGTGGCRTATCCTTCCC-3′
		5′-GACRATACATTGGGAAGGATAYGCCACAAACATAATGGTT-3′
MSH2	*rs2303425*	5′-ACCCWCSKAAACSCAGCCCTGGAAGC-3′
		5′-KACCAYAYCMARTCAGCTTCCAGGGCTGSGTTTMSGWGGGT-3’

Brazil, with 92% of its territory located between the equator and the Tropic of Capricorn, receives a higher incidence of UVB rays. Being the geographical location of the state of Paraíba propitious to exposure of high levels of said rays [5].

UVB radiation penetrates into the epidermis is considered important physical agent involved in the process of cutaneous carcinogenesis, because it can lead to DNA damage [6]. Cells present alternative DNA repair pathways to reverse different lesions caused by both endogenous and exogenous agents [7].

The Mismatch Repair - MMR is conserved in both eukaryotes and prokaryotes [8]. Eukaryotic systems utilize two groups of proteins homologous to those of prokaryotes. MSHs that function as heterodimers of two distinct subunits Mutsα (MSH2-MSH6) that recognize single nucleotides unequal pairing and Mutsβ (MSH2-MSH3) recognize insertion / deletion loops and form part of this MSH a complex (Msh4-Msh5), which have no function in the MMR system, but are important during mitotic recombination, since they recognize Holliday joints [9].

The MLH proteins function as heterodimers (MutLα: MLH1-PMS2; MutLβ: MLH1-PMS1; MutLy: MLH1-MLH3). Thus all cited proteins are of extreme importance for proper function of the MMR pathway in humans [10].

In humans the MMR is first recognized by two MSH2-MSH6 or MSH2-MSH6 heterodimers, which when linked recruit the MLH1-PMS2 complex, which has an endonuclease site in PMS2, it is also essential to recruit other proteins for repair as Exon1, PCNA, RPA and the DNA Polymerase δ [11, 12].

The MLH1 gene in humans, located in the chromosomal region 3p22.2, consists of 19 exons, being 58Kb in length, which encodes a nuclear protein with 756aa having a molecular weight of 80 KDa [13, 14].

The MSH2 gene is also involved with MMR, which is located in humans in the chromosomal region 2p21, which covers a 73Kb segment with 16 exons, encoding a nuclear localization protein containing 934aa, having molecular weight corresponding to 104,7 KDa [15, 16].

Polymorphic mutations in the genes of the MMR repair mechanism, especially in the hMLH1 and hMSH2 genes affecting both are a risk factor for the susceptibility to the development of hereditary and sporadic tumors [17–21].

The present study analyzed the unique nucleotide polymorphisms (SNPs) rs560246973 (T > C), rs2303425 (−118 T > C) in the MSH2 gene and rs565410865 (G > T) in the MLH1 gene in paraffined tissue samples from patients diagnosed with carcinoma in the state of Paraíba in northeastern Brazil.

Material and Methods

Samples

The present study analyzed 100 samples of paraffin-embedded tissue from patients with histopathological diagnosis of BCC, totaling 200 alleles for each SNP studied and 100 control samples obtained from paraffin-embedded normal tissue from cancer-free patients from the Laboratory of Structural Molecular Biology and Oncogenetic – LABMEO/ UFPB sample bank.

It is a retrospective study and samples has more than five years old and were provided by the bank of data by the Laboratory UNILAB/João Pessoa – PB and LABMEO/ UFPB sample bank. The present study is part of the thematic project approved by the Ethics Committee of the University Hospital Lauro Wanderley - UFPB under the code CAAE: 36,522,614.2.3001.5883.

DNA Extraction

The samples were submitted to DNA extraction in Laboratory of Structural Molecular Biology and Oncogenetic - LBMEO/

Table 2 Genotypic distribution of SNPs in the genes studied in BCC and control samples

Samples	Gene/SNP	Genotypic distributions			X^2	p-valor
	MSH2/rs2303425	C/C	C/T	T/T	2,25	0,32
BCC		82	18	0		
Control		83	16	1		
	MSH2/rs560246973	T/T	T/C	C/C	96,73	0,0001
BCC		78	0	22		
Control		61	34	5		
	MLH1/rs565410865	G/G	G/T	T/T	86,13	0,0001
BCC		84	0	16		
Control		70	27	3		

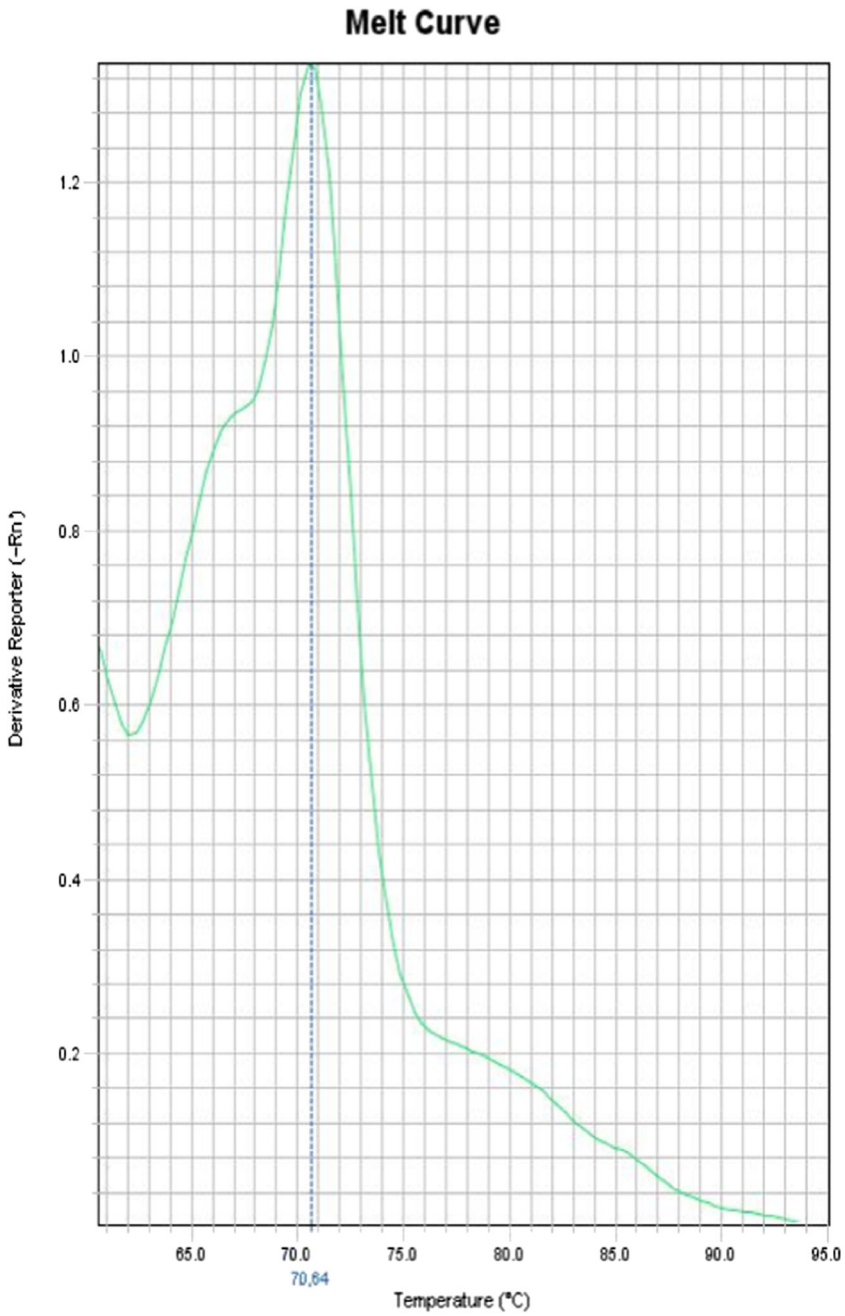

Fig. 1 Melting curve representing the T/T genotype of the SNP rs560246973 of the MSH2 gene

UFPB. The DNA sample was extracted according to the method described by Shang Rong-Shi et al. [22] with modifications. The isolated genomic DNA was stored at -20 °C.

Method Dideoxy Single Allele-Specific PCR-DSASP

The DSASP method is based on chain-terminating inhibitors, dideoxynucleotide, established by the method of Sanger et al. (1977). DSASP was previously validated by the Allele Specific PCR-ASP method as described by Lima et al. [23].

The DSASP is a method of genotyping and comprises four stages: I - selection of SNPs of interest, design of the oligonucleotides (primer and complementary sequence) and determination of the dideoxynucleotide to be incorporated; II - asymmetric PCR as dideoxynucleotide of interest; III - hybridization reaction between complementary sequence and asymmetric PCR product; IV - analysis by melting curve by qPCR and was validated and developed by Lima et al. [23].

To genotype the *SNPs* rs560246973 (T > C) and rs2303425 (−118 T > C) (MSH2 gene) and rs565410865 (G > T) (MLH1 gene) by DSASP method, asymmetric PCR was performed for each SNP of interest, complementary sequence and dideoxynucleotide incorporation. The oligonucleotides were obtained by in silico validation (GeneRunner Software).

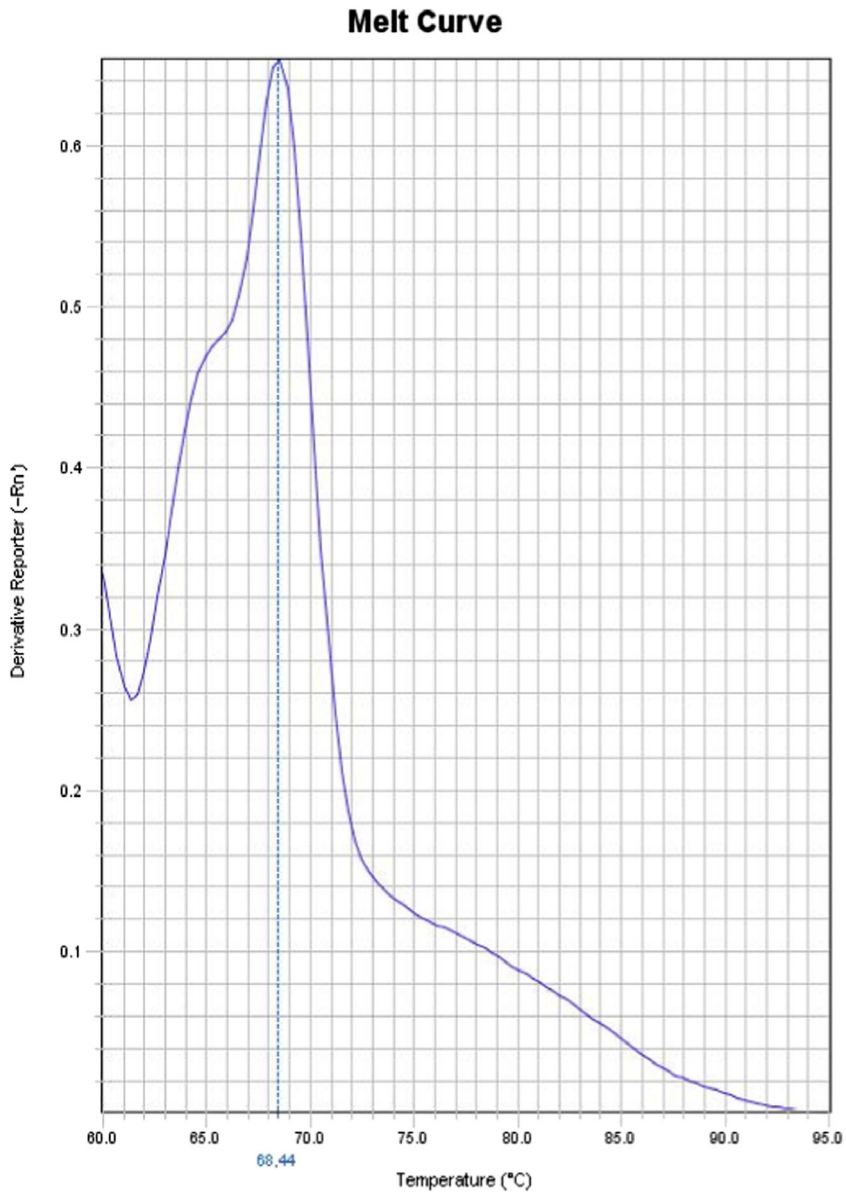

Fig. 2 Melting curve representing the C/C genotype of the SNP rs560246973 of the MSH2 gene

Validation in Silico

The primers used for DSASP of the *SNPs* rs560246973 (T > C) and rs2303425 (−118 T > C) (MSH2 gene) and rs565410865 (G > T) (MLH1 gene) were designed based on the database Ensembl Genome Browser and using the Gene Runner program (Table 1) to evaluate the annealing temperature, the formation of secondary structure and size of the amplified fragments.

Asymmetric PCR Conditions

Asymmetric PCR was performed in a final volume of 25 μL containing 200 μM dNTP (dATP, dCTP, dTTP and ddGTP), 2.0 mM $MgCl_2$, 20 ng/μL DNA, 200 *p*M primer and 0.5 U AmpliTaq Gold (Life Technologies - Carlsbad, CA). PCR conditions for amplification of single stranded DNA were as follows: a pre-denaturation for 3 min at 94 °C and 80 cycles of 94 °C for 20 s of 50 °C for 45 s and 72 °C for 30 s with a final extension of 5 min at 72 °C.

Hybridization Conditions

The product of the PCR amplification of each sample was subjected to hybridization step under the following conditions: 200 *p*M of the complementary sequence at 4 °C for 10 min.

Melting Curve Analysis

The melting curve were analysed to determine the Tm performed by Real Time PCR equipment 7500 Fast Real-Time

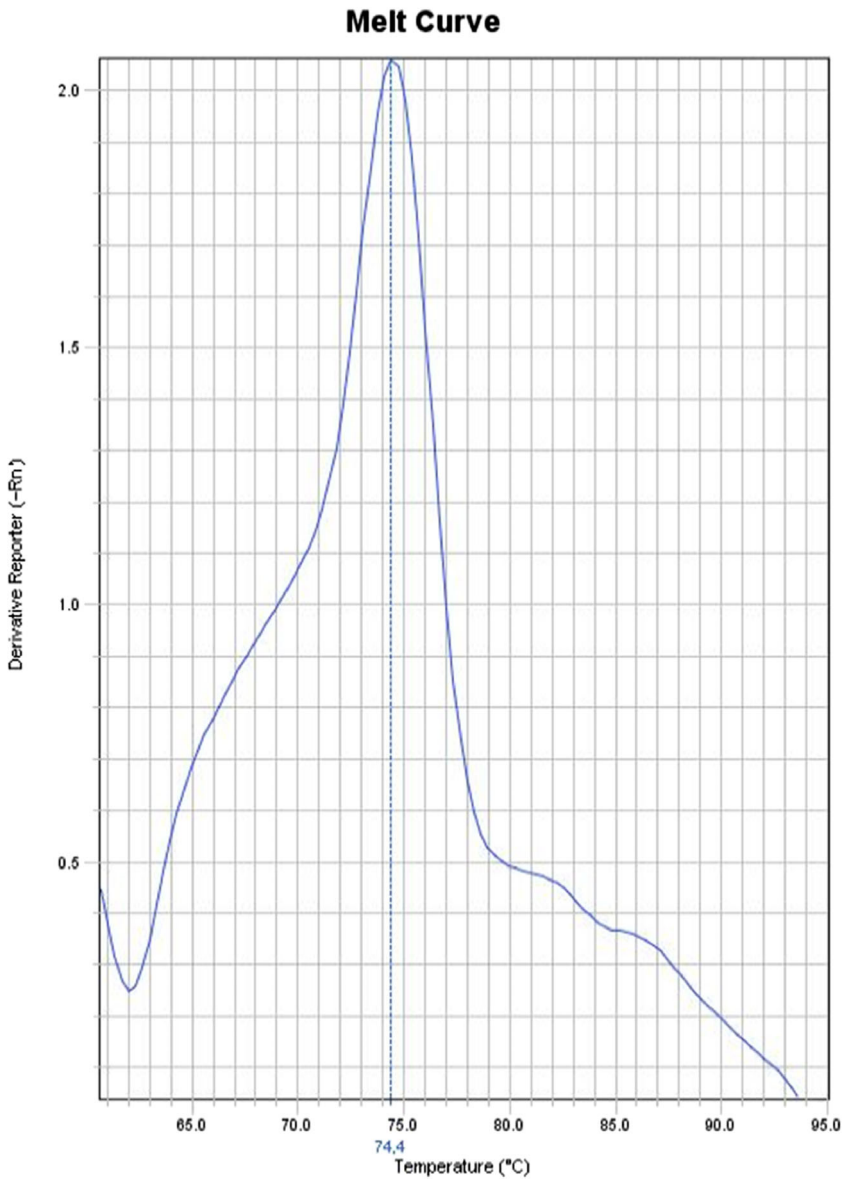

Fig. 3 Melting curve representing the G/G genotype of the SNP rs565410865 of the MLH1 gene

PCR System (Life Technologies - Carlsbad, CA) following the conditions: preheat starting at 25 °C to 95 °C for 1 min, folding up to 45 °C for 5 min and gradual heating (1 °C/min) until a temperature of 95 °C for 5 min. For melting curve analysis, SYBR® Green Mix (Life Technologies - Carlsbad, CA) was used.

Statistical Analysis

The allelic frequencies and genotypic distributions were obtained by the Hardy-Weinberg equilibrium model. The association analysis was performed using Chi-square and Fisher's exact test, using the statistical program BioEstat 5.3, where $P < 0.05$ was considered significant.

Results

The SNPs rs2303425 (−118 T > C) of the MSH2 gene were not associated with Basal Cell Carcinoma. However, the SNPs rs560246973 (T > C) of the MSH2 gene and rs565410865 (G > T) *MLH1* gene was shown to be associated with the development of BCC when compared to control samples ($P < 0.0001$) (Table 2). The genotypic distributions of rs560246973 (T > C) of the MSH2 gene was $n = 77$ (T/T), $n = 23$ (C/C) and $n = 0$ (C/T) and was the observed Tm 70 °C for allele A and 87 °C for allele C (Figs. 1 and 2).

The genotypic distributions of rs565410865 (G > T) of the MLH1 gene was $n = 87$ (G/G), $n = 13$ (T/T) and $n = 0$ (G/T) and was the observed Tm 74 °C for allele G and 75 °C for allele T (Figs. 3 and 4).

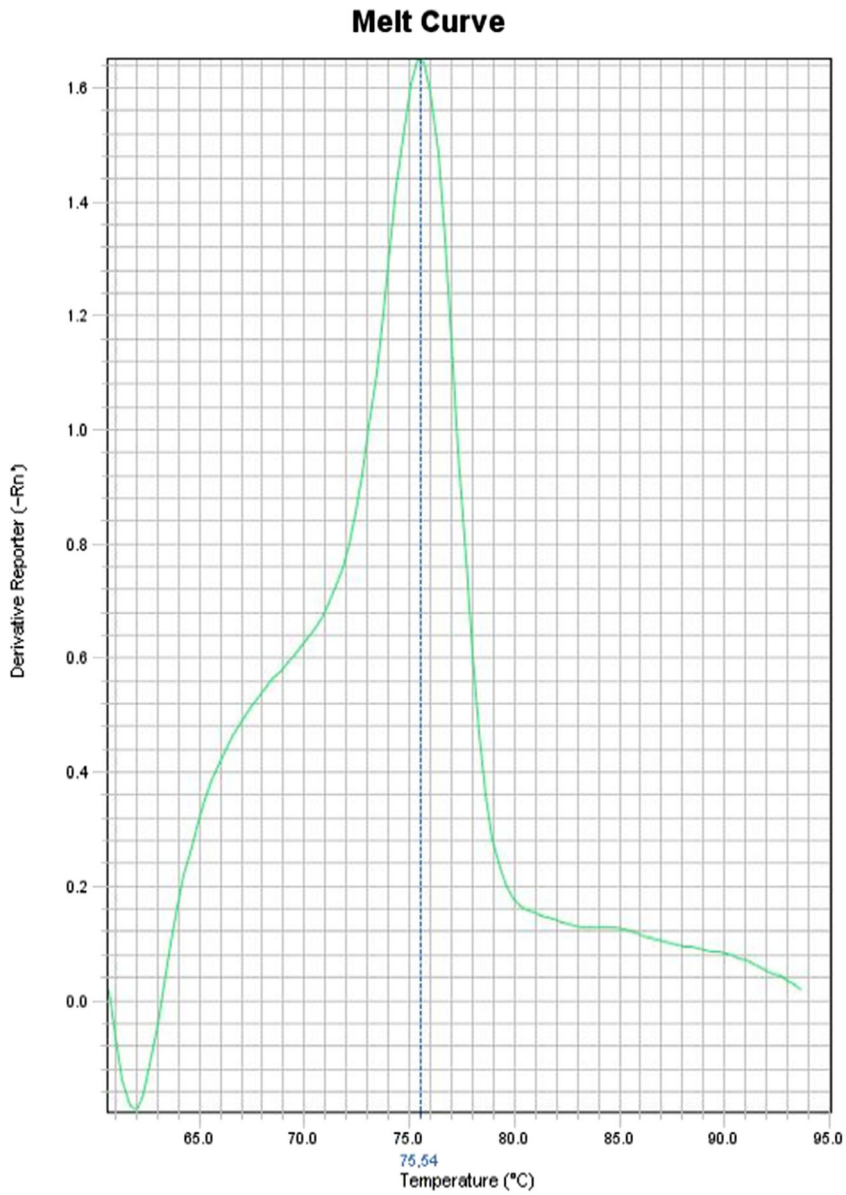

Fig. 4 Melting curve representing the T/T genotype of the SNP rs565410865 of the MLH1 gene

Our results also suggest that there was a statistical significance between the genotypes of the MLH1 gene rs565410865 (G > T) and the age group (p = 0.0027) and tumor location (p = 0,0191).

Discussion

Several single-nucleotide polymorphisms have been associated with susceptibility to the development of cancer. Genetic alterations in genes that participate in the MMR pathway, even in intron regions, especially MLH1 and MSH2 genes may be associated with neoplastic phenotype, as they may affect the normal function of the protein [24, 25]. The SNPs investigated in this work (rs560246973, rs2303425 MSH2 gene and rs565410865 MLH1 gene) is the first time they are described and evaluated in basal cell carcinoma in the Brazilian population.

Analysis of the SNP rs2303425 (−118 T > C) in the promoter region of the MSH2 gene in BCC samples showed no significant association. Our results corroborate the findings described by Srivastava and Mitta (2010) in a North Indian patient with gallbladder carcinoma, where they show that there was no significant association of the SNP rs2303425 with the development of the carcinogenesis of this neoplasia [26] and study of control cases performed by Xiao et al. (2012) in gastric cancer in China has not observed association of SNP rs2303245 with the development of gastric cancer [17].

However, Hsieh et al. (2017) suggest that the C/C genotypes of rs23032425 present an increased risk of breast cancer, as well as decreased MSH2 expression compared to T/T genotypes,

suggesting a decrease in promoter activity, being a potential molecular marker for cancer In the Asian population [27].

A likely explanation for the association of the rs2303425 SNP to breast cancer is the change in the binding site of the transcription factor NF-Y, a potent estrogen regulatory element. The C allele creates an AP1 binding site that is activated by antiestrogens, so it can alter the responsiveness of the MSH2 promoter to estrogen and decrease MMR activity in endometrial cells [20, 28–30].

However, the SNPs rs565410865 in the MLH1 gene and SNP rs560246973 in the MSH2 gene analyzed in the present study had a significant association with the susceptibility and risk of developing BCC. The SNPs are located in the intersection region of exon and intron. Studies suggest that substituting base into a DNA sequence in an intron region may alter gene regulation at the post-transcriptional level as it may create new processing sites as well as alter regulatory splicing elements [31, 32].

These regulatory elements contribute to the fidelity of pre-mRNA processing because they are located within exons and introns at specific sites for the recognition of spliceosome and RNA-binding auxiliary proteins at the correct sites for introns removal and the junction of exons [33].

The results are corroborated by Zahary et al. (2012), in which they reported a significant decrease in the expression of the MSH2 protein, consequently associated with a mutation in a splicing site, in which it could result in the compromise of the exon 13, most conserved region of the MSH2 gene [34].

As well as the population study performed by Langerberg et al. (2010) established an association of the SNP rs9852810 (G > A) located in an intron region of the MLH1 gene, with moderate risk in the development of prostate cancer [35].

The SNPs rs560246973 and rs565410865 was shown to be associated with the development of BCC when compared to control samples and the SNPs rs565410865 was also statistical significance between the genotypes of and the age group and tumor location. Therefore, the result suggests that SNPs rs2303425 and rs565410865 are associated with susceptibility to the development of BCC in the Brazilian population and may be considered as potential molecular markers for BCC.

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Compliance with Ethical Standards

Conflict of Interest None.

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Ethical Approval The present study is part of the thematic project approved by the Ethics Committee of the University Hospital Lauro Wanderley - UFPB under the code CAAE: 36,522,614.2.3001.5883.

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