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3'UTR-CDKN2A and CDK4 Germline Variants Are Associated With Susceptibility to Cutaneous Melanoma

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Abstract. *Background/Aim: Genetic variations of the CDKN2A and CDK4 gene have been associated to melanoma development. In the present study we investigated the potential associations of CDKN2A and CDK4 gene variants in a colombian population diagnosed with melanoma. Materials and Methods: DNA was extracted from whole blood samples from 85 patients diagnosed with cutaneous melanoma and 166 healthy controls. CDKN2A and CDK4 genes were genotyped using a high-resolution melting assay. Results: A similar distribution of CDKN2A variants 500C>G and 540C>T was found among cases (12% and 31% respectively) and controls (15% and 31% respectively). The CDKN2A variants were present in 36% of acral lentiginous melanoma and 39.47% of lentigo maligna. The haplotype analysis showed an association with susceptibility in the development of melanoma. Conclusion: The presence of haplotype 500G/540C in males is associated with an increased risk of melanoma in a colombian population, especially in subjects with a family history of cancer.*

Approximately 324,635 cases of melanoma are diagnosed annually worldwide with a mortality rate of 22% (1). Melanoma etiology is complex and heterogeneous, involving environmental and genetic factors such as sun exposure, skin color, sex, number, and types of nevi, specific germline variants, and somatic mutations. CDKN2A and CDK4 genes exhibit high penetrance variants associated with melanoma pathogenesis (2, 3). Approximately 10% of patients with melanoma who report a positive family history of melanoma have germline pathogenic variants. However, in patients with cutaneous melanoma without positive family history of melanoma, the germline pathogenic variants are usually rare but their presence may increase the risk of melanoma development (4).

CDKN2A gene encodes the protein p16, which inhibits the activity of the CDK4-cyclin complex. This complex phosphorylates the retinoblastoma protein allowing the cell to progress through G_1 phase. Besides that, another protein encoded by CDKN2A is p14ARF(ARF), which regulates critical cell-cycle pathways, and it also inhibits MDM2 that leads to ubiquitination of p53 (5, 6).

CDK4 gene encodes the catalytic subunit of a heterodimeric Ser/Thr protein kinase, which is involved in the control of the cell cycle (5). Germline variants on the CDK4 gene have been associated with an increased risk of melanoma development. These variants are located in codon 24 in exon 2, affecting the binding site with p16 protein and therefore promoting cell-cycle progression (7). For example, p.R24C and p.R24H variants of CDK4 lead to a 50-fold increased risk of developing melanoma (8, 9).

The p16-cyclin D-CDK4/6-retinoblastoma protein (RB1) signaling (CDK4 pathway) is commonly dysregulated in various types of cancers (10). CDKN2A variants such as 500C>G (rs11515) and 540C>T (rs3088440, located in the 3'UTR: untranslated gene region), affect mRNA stability and post-transcriptional regulation of gene expression for both p16 and p14 proteins. Additionally, p.R24C (rs11547328) and p.R24H (rs104894340) variants of CDK4 affect its binding with p16 protein. For that reason, several studies have been conducted to estimate their association with melanoma development (11-13).

In the present study, we evaluated the association between four CDKN2A and CDK4 variants and the development or susceptibility of cutaneous melanoma in a Colombian population and we described the frequencies of these variants in a complex genetic background of our populations.

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Key Words: Haplotype, melanoma, cancer, variant, susceptibility, germline variant.

Table I. *Primer's pairs used in the conventional PCR reaction and HRM-qPCR reaction.*

Conventional PCR

Gene	Exon variants	Primers 5'-3'	Product size (pb)	Melting temp. (˚C)	Nucleotide sequence 5'-3'
CDKN2A	3	F	328	58.4	CCATTGCGAGAACTTTATCC
		R			TGGACATTTACGGTAGTGGG
CDK4	2	F	274	58.2	CTGTAAGCGACTTTTGGTGATAG
		R			GACAACATTGGGATGCTCA

HRM – qPCR assay

Gene	rs	SNP	Protein Change	Primers 5'-3'	Product size (pb)	Melting temp. (˚C)	Nucleotide sequence 5`-3`
CDKN2A	rs11515	500C>G		F	138	59	CTGTAGGACCTTCGGTGACTG
				R			TGTGCCACACATCTTTGACC
	rs3088440	540 C>T		F	153	59	TTAGATCATCAGTCACCGAA
				R			GGACATTTACGGTAGTGGGG
CDK4	rs11547328	c.70C>T	p.R24C	F	236	59	TTGGTGATAGGAGTCTGTGA
	rs104894340	c.71G>A	p.R24H	R			TCCAGTCGCCTCAGTAAAGC

Materials and Methods

Study participants. The case and control study included 85 melanoma patients and 166 healthy controls, who were recruited between 2018 and 2019 at the Hospital Universitario Centro Dermatológico Federico Lleras Acosta E.S.E in Bogotá D.C, Colombia. Written informed consent was obtained for each patient included in the study. For cases group, the inclusion criteria considered age greater than 18 years and patients diagnosed with cutaneous melanoma. For control group, inclusion criteria included age greater than 18 years and subjects with neither family history of melanoma nor personal history of melanoma. Demographic, morphological, and clinical data was collected during the recruitment period. Variables such as family history of melanoma, history of cancer, nevus count, Fitzpatrick phototype, hair and eyes color were documented.

Institutional Review Board statement. The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Research Ethics Committee of the Hospital Universitario Centro Dermatológico Federico Lleras Acosta E.S.E, Bogotá, Colombia. (Grant code 4000.16.6AE).

Genomic DNA extraction and genotyping assays. Blood samples were obtained from 251 subjects (85 cases and 166 controls) between years 2018 and 2019. Genomic DNA was extracted from patient's using a QIAamp® DNA Mini and Blood Mini Handbook kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. After quantitation using UV-Vis spectrometry, DNA samples were stored at –20˚C for future use. PCR conditions were described in the previous study (14). CDKN2A and CDK4 primers are available in Table I.

Variant identification of the rs11515, rs3088440, rs11547328 and rs104894340 were carried out by High Resolution Melting analysis (HRM), using CFX96 Touch Tm Real-Time PCR detection system (Bio-Rad, Laboratories, Hercules, CA, USA). The samples were processed with Precision Melt Supermix kit (Bio-Rad, Laboratories). The real-time PCR was conducted in a total volume of 10 ul, containing 20 ng of genomic DNA and 0.5 μM of each primer. The sequence of primers for HRM analysis is listed in Table I. All samples were tested in duplicate. The conditions of qPCR and HRM analysis were: initial denaturation at 95˚C for 2 min; 45 cycles of denaturation at 95˚C for 10 sec, annealing for 30 s (between 53˚C and 65˚C depending on the exon) (Table I), and 72˚C extension cycle for 30 s. To induce heteroduplex formation, the PCR products were subjected to heating at 95˚C for 30 s, at 60˚C for 1 min, and HRM cycle at 65-95 ºC for 10 s/step in 0.2˚C increments.

Wild type and mutant DNA, and non-template were used as controls groups in each amplification test. The data was analyzed using the Precision Melt Analysis™ v1.1 Software (Bio-Rad), according to the controls wild type, homozygous or heterozygous. The samples were assigned to different groups. Accuracy of HRM genotypic procedure was confirmed by direct DNA sequencing from several randomly selected samples. The primers of conventional PCR were used to amplify the DNA in the sequencing. The length of the amplicons is listed in Table I. The sequence was performed according to standard methods for Sanger sequencing using a BigDye Terminator V1.1 Cycle Sequencing Kit and ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems®). Variant analysis was performed using the software Chromas (free version). All sequences were confirmed by a second researcher independently (Figure 1).

Statistical analysis. Stata 16® was used for the Statistical analysis. Hardy Weinberg Equilibrium (HWE) was tested comparing genotype frequencies in both groups using Chi-square test (χ^2). Shapiro Wilk test or Wilcoxon rank sum test was used to determinate the normal distribution for each variable. SNPStats program (Institut Catalá d'Oncologia, Barcelona, Spain), Chi-square test and Fisher's test were applied to determine difference between genotypes, haplotypes, or allele. The genetic association was evaluated calculating Odds Ratio (OR) with a 95% confidence interval, statistical significance was defined as $p<0.05$. R-Studio was employed to confirm Haplotypes, with the Haplo.Stats package version 1.7.7 (Haplotype Score Tests or Regression Models).

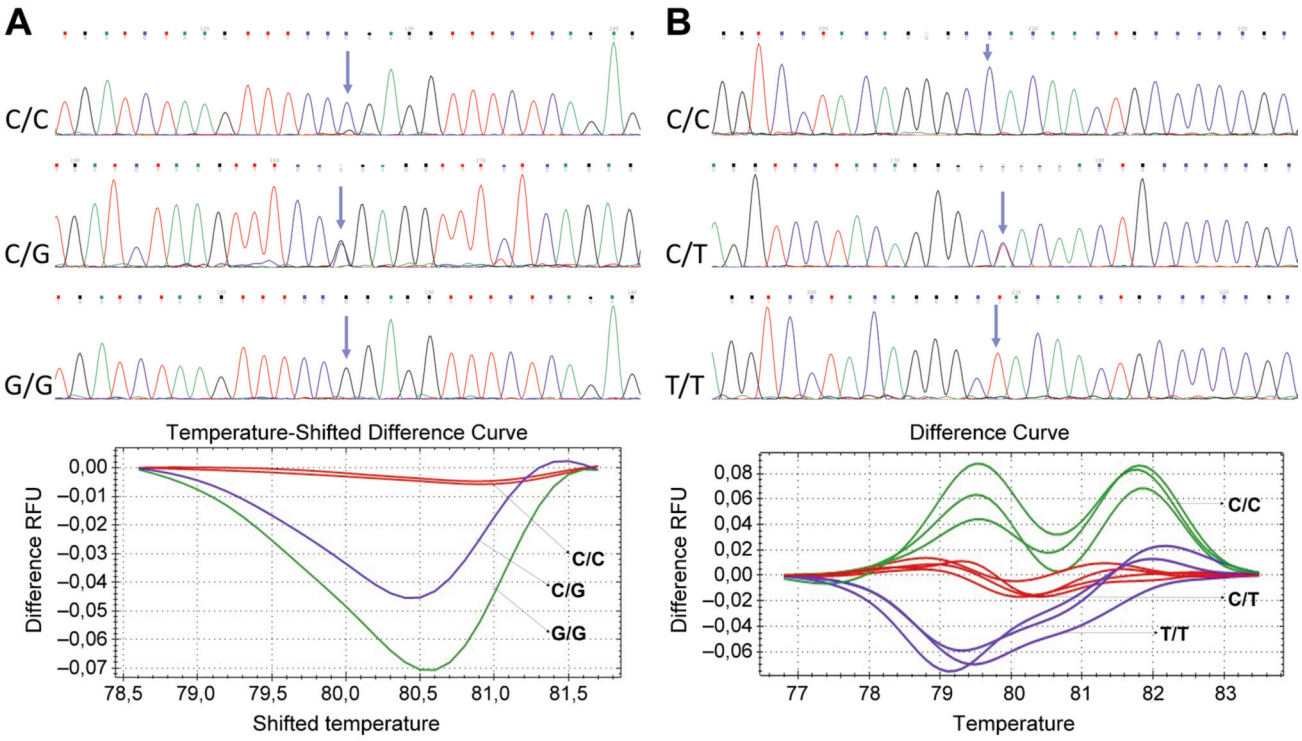

Figure 1. *Identification of 500C>G and 540C>T variants by High-resolution melting (HRM) and sequencing in CDKN2A gene. A). Sequencing and differences curves by HRM, for identification of 500C>G variant. B). Sequencing and differences curve by HRM for identification of 540C>T variant in CDKN2A gene.*

Results

A total of 85 melanoma patients and 166 healthy individuals were included in the study. Demographics and clinical characteristics of cases and control groups was reported in previous studies (14).

Association between CDKN2A and CDK4 genetic variations and melanoma risk. The genotypic results for CDKN2A 500C>G (rs11515), 540C>T (rs3088440) and CDK4 p.R24C (rs11547328), p.R24H (rs104894340) variants, are shown in Table II. Two genetic variants, rs11515 and rs3088440, were found to be in HWE in the control participants ($p<0.05$). Our results showed that polymorphisms of all candidate SNPs were not associated with melanoma risk ($p>0.05$) (Table II).

CDKN2A variants 500C>G and 540C>T were present in 12% (11/85) and 31% (38/85) for cases and 15% (26/166) and 31% (69/166) for controls, respectively. The 500C>G variant was homozygous in 12% of the cases and 15% of the controls, meanwhile, the 540C>T variant was homozygous in 18% of the cases and 20% of the controls. On the other hand, CDK4 gene did not present variants in either case and control groups. Four types of genetic models (co-dominant, dominant, recessive and overdominant) were used in the association analysis of CDKN2A variants with susceptibility to develop melanoma (Table II). Our results indicate that p.R24C and p.R24H variants in the CDK4 gene were not present in either group, and 500C>G and 540C>T variants in CDKN2A gene did not display any statistical differences between case and control groups.

Regarding the 500C>G and 540C>T variants, 72.73% and 55.26% were women, respectively. Besides that, according to melanoma subtype, 36.36% were acral lentiginous melanoma (ALM) and 27.27% were Lentigo Maligna Melanoma (LMM) with 500C>G variant. 540C>T variant was present in 26.32% of ALM and 39.47% of LMM. Five cases and seven controls displayed 2 variants, where 4/5 were women in cases and 3/7 were women in control groups. For the 500C>G variant, 45.45% of cases presented ulceration, conversely 18.42% with 540C>T variant presented ulceration. According to the location of melanoma, the cases that presented the 500C>G variant were in head and neck, and lower extremities (36.36%), and cases with 540C>T variant were located mainly in head and neck (47.37%).

According to the Fitzpatrick phototypes, individuals that have 500C>G variant were phototype II in 63.64% of the cases and 65.38% of control group. Those with the 540C>T variant were phototype II in 73.68% of the cases and 72.46% of control group. Furthermore, 72.73% of the cases with 500C>G variant and 60.53% of the cases with 540C>T variants had a family history of melanoma (Table III).

Table II. *Genotype and allele distributions of variants CDKN2A and CDK4 gene in cases and controls groups.*

Model	Genotype Allele	Cases n=85 (%)	Control n=166 (%)	OR	(95% CI)	*p*-value
CDKN2A (rs11515; 500-3´UTR)						
Codominant						
	C/C	74 (87.1%)	139 (84.2%)	1	Reference	
	C/G	1 (1.2%)	3 (1.8%)	NA	NA	0.23
	G/G	10 (11.8%)	23 (13.9%)	1.51	(0.60-3.81)	0.23
Dominant	C/C	74 (87.1%)	139 (84.2%)	1.72	(0.70-4-24)	0.23
	C/G+G/G	11 (12.9%)	26 (15.8%)			
Recessive	C/C+C/G	75 (88.2%)	142 (86.1%)	1.48	(0.59-3-73)	0.4
	G/G	10 (11.8%)	23 (13.9%)			
Overdominant	C/C+G/G	84 (98.8%)	162 (98.2%)	NA	NA	0.14
	C/G	1 (1.2%)	3 (1.8%)			
	C	21 (12)	49 (15)	0.808	(0.46-1.39)	0.446
CDKN2A (rs3088440; 540-3´UTR)						
Codominant	C/C	47 (55.3%)	96 (58.2%)	1	Reference	
	C/T	23 (27.1%)	36 (21.8%)	0.73	(0.35-1.49)	0.34
	T/T	15 (17.6%)	33 (20%)	1.48	(0.64-3.44)	
Dominant	C/C	47 (55.3%)	96 (58.2%)	0.98	(0.53-1.79)	0.93
	C/T+T/T	38 (44.7%)	69 (41.8%)			
Recessive	C/C+C/T	70 (82.3%)	132 (80%)	1.62	(0.71-3.66)	0.24
	T/T	15 (17.6%)	33 (20%)			
Overdominant	C/C+T/T	62 (72.9%)	129 (78.2%)	0.67	(0.33-1.34)	0.26
	C/T	23 (27.1%)	36 (21.8%)			
	T	53 (31)	102 (31)	1.012	(0.678-1.510)	0.951
CDK4 (rs104894340; p.R24H)						
	C/C	85 (100)	166 (100)	1	Reference	
	C	170 (100)	332 (100)			
CDK4 (rs11547328; p.R24C)						
	G/G	85 (100)	166 (100)	1	Reference	
	G	170 (100)	332 (100)			

Association between haplotypes and melanoma risk. The most prevalent haplotype combination was 500C-540C in CDKN2A gene, which was present in 59% of cases and 57% of controls, but this analysis did not present any statistical significance between cases and control groups. However, when haplotype interaction analysis with sex and family history cancer was performed, an association was shown in males with haplotype 500G/540C when having a family history of cancer OR=2.76 (95% CI=1.01-7.52) (Table IV).

Discussion

Due to the low melanoma incidence in the Latin-American region, few studies have been carried out, compared to other populations around the world. An interesting aspect is that reported subtypes of melanoma in the Latin-American region are different to those reported in high-incidence regions. In the Colombian population, several studies have shown that lentigo malignant melanoma is the most frequent with 36.41-52%, followed by acral lentiginous melanoma with 20-24.71% incidence (15). The frequency of melanoma in Latin America varies according to the studied population. For example, in the Argentinian population, the frequencies reported are 33.8% for superficial spreading and 14.5% for nodular melanoma (16) while in Brazil these are 34.45% for superficial spreading and 38.9% for nodular melanoma (17), suggesting that there may be genetic and environmental differences leading to various types of melanomas in this region.

Germline alterations in CDKN2A and CDK4 gene in DNA samples of cases and controls were analyzed to investigate known cancer susceptibility gene variants. The results showed that case and control groups did not display

Table III. *Demographic and clinical characteristics of cases and controls groups with 500C/G and 540C/T variants in CDKN2A gene*.

Feature	CDKN2A							
	500C>G rs11515				540C>T rs3088440			
Cases=85	Cases		Controls		Cases		Controls	
Controls=166	11	%	26	%	38	%	69	%
Gender								
Women	8	72.73	11	42.31	21	55.26	36	52.17
Men	3	27.27	15	57.69	17	44.74	33	47.83
Melanoma subtype								
Superficial spread	3	27.27	0	0	5	13.16	0	0
Nodular	1	9.09	0	0	8	21.05	0	0
Acral lentiginous	4	36.36	0	0	10	26.32	0	0
Lentigo maligna	3	27.27	0	0	15	39.47	0	0
Clark level								
Negative	6	54.55	0	0	14	36.84	0	0
I	0	0	0	0	2	5.26	0	0
II	0	0	0	0	4	10.53	0	0
III	0	0	0	0	0	0	0	0
IV	3	27.27	0	0	16	42.11	0	0
V	2	18.18	0	0	2	5.26	0	0
Breslow scale								
Non reported	5	45.45	0	0	15	39.47	0	0
≤1. 0 mm	1	9.09	0	0	4	10.53	0	0
>1.0-2.0 mm	1	9.09	0	0	4	10.53	0	0
>2.0-4.0 mm	1	9.09	0	0	4	10.53	0	0
>4.0 mm	3	27.27	0	0	11	28.95	0	0
Mitoses								
Non reported	5	45.45	0	0	15	39.47	0	0
Negative	1	9.09	0	0	5	13.16	0	0
≤1.0 mm^2	2	18.18	0	0	7	18.42	0	0
>1.0 mm^2	3	27.27	0	0	11	28.95	0	0
Ulceration								
Non reported	4	36.36	0	0	15	39.47	0	0
Presence	5	45.45	0	0	7	18.42	0	0
Absence	2	18.18	0	0	16	42.11	0	0
Location								
Trunk	1	9.09	0	0	5	13.16	0	0
Head and Neck	4	36.36	0	0	18	47.37	0	0
Upper extremities	0	0	0	0	2	5.26	0	0
Lower extremities	2	18.18	0	0	3	7.89	0	0
Hands and Feet	4	36.36	0	0	10	26.32	0	0
Phototype								
II	1	9.09	6	23.08	6	15.79	9	13.04
III	7	63.64	17	65.38	28	73.68	50	72.46
IV	3	27.27	3	11.54	4	10.53	10	14.49
Eye color								
Black or dark brown	7	63.64	18	69.23	22	57.89	59	85.51
Light brown	3	27.27	4	15.38	11	28.95	9	13.04
Green	1	9.09	2	7.69	4	10.53	1	1.45
Blue	0	0	2	7.69	1	2.63	0	0
Hair color								
Black or dark brown	8	72.73	22	84.62	23	60.53	59	85.51
Light brown	3	27.27	4	15.38	15	39.47	9	13.04
Red or blond	0	0	0	0	0	0	1	1.45
Personal history of cancer								
Yes	1	9.09	1	3.85	0	0	1	1.45
No	10	90.91	25	96.15	38	100	68	98.55

Table III. *Continued*

Table III. *Continued*

Feature	CDKN2A							
	500C>G rs11515				540C>T rs3088440			
Cases=85	Cases		Controls		Cases		Controls	
Controls=166	11	%	26	%	38	%	69	%
Family history of cancer								
Yes	8	72.73	12	46.15	23	60.53	35	50.72
No	3	27.27	14	53.85	15	39.47	34	49.28
Personal history of melanoma								
Yes	0	0	0	0	4	10.53	0	0
No	11	100	26	100	34	89.47	69	100
Family history of melanoma								
Yes	1	9.09	0	0	4	10.53	1	1.45
No	0	0	26	100	34	89.47	68	98.55
Nevus								
≤50	10	90.91	26	100	32	84.21	68	98.55
50-100	1	9.09	0	0	5	13.16	1	1.45
>100	0	0	0	0	1	2.63	0	0

Table IV. *CDKN2A haplotype frequencies and the association with melanoma risk.*

Haplotype frequencies estimation (n=250)						
N°	500C>G	540C>T	Total	Group.Ca*	Group.Co**	Cumulative frequency
1	C	C	0.5828	0.5966	0.5758	0.5828
2	C	T	0.2772	0.2798	0.2758	0.86
3	G	C	0.1072	0.0916	0.1152	0.9672
4	G	T	0.0328	0.0319	0.0333	1

Haplotypes within sex (n=250, adjusted by family)			
Haplotype	Frequency	Female OR (95% CI)	Male OR (95% CI)
CC	0.5828	1.00	1.00
CT	0.2772	1.21 (0.66-2.23)	1.17 (0.65-2.10)
GC	0.1072	0.85 (0.40-1.80)	2.76 (1.01-7.52)
GT	0.0328	0.91 (0.28-2.96)	1.74 (0.31-9.56)

any germline variants in CDK4 gene (p.R24C and p.R24H). A study analyzed an Australian population of 1,109 melanoma patients, but no CDK4 variants were found (18). However, prior studies reported that CDK4 variants frequencies are approximately between 2-15% in Greek (19), Latvian (20), and other populations (5).

Germline variants (500C>G and 540C>T) in the CDKN2A 3'UTR gene have been found in approximately 12-31% of cutaneous melanomas and healthy controls, respectively (21-23), as well as associated with a risk of developing other types of cancer (24). However, it was shown in previous reports, that 14% of patients of 30 Israel melanoma families presented 500C>G germline variants, and other germline variants (25). A total of 285 patients analyzed in a Polish population reported that 21.78% of them presented the 500C>G variant while the 540C>T variant was not found (26), but in an Italian population Pellegrini *et al.* in 2017, reported frequencies of 38% for the 500C>G variant and 16% for the 540C>T variant (23). Consequently, those variants in CDKN2A 3'UTR were associated with high-risk melanoma in different case-control studies and have been reported the 500C>G and 540C>T variants in CDKN2A gene as a pathogenic gene alteration (21, 27). Maccioni *et al.* in 2013 reported association of 540C>T variants with melanoma risk, OR=1.52 (95%CI=1.14-2.04)

(28), and Kumar *et al.* in 2001 reported an OR=1.71 (95%CI=1.40-3.92) in Sweden population (22).

It was found that 5/11 cases presented both variants (500C>G and 540C>T), where 4/5 were women, and 100% had family history of cancer. The presence of both variants have been reported to be associated with a shorter disease-free survival and poor prognosis (22). The present and other studies confirm that the frequencies of CDKN2A 3'UTR variants depend on different factors, such as selection criteria, melanoma incidence in population analyzed, genetic ancestry and number of patients included in the study. This study reports that the incidence rate of germline variants of females was between 55-72% in cases, and 42-52% in controls groups. Furthermore, it was also found that 60-72% of cases, and 46-50% of controls had a history of other types of neoplasms (22). Multiple studies around the world have evaluated melanoma high-risk genes in patients with family history of melanoma and have reported heterogeneous results for the genetic variants association with the development of melanoma (20, 22, 23, 26, 29).

Those variants in CDKN2A 3'UTR had been associated with susceptibility to develop melanoma, in different case and control studies. But they have not been associated with the clinical features of patients or pathological characteristics of melanoma (22). In the present study, the haplotype distribution analysis reported no significant difference in distribution between cases and controls groups. However, it is estimated by haplotype interaction analysis with sex and family history of cancer, that an association exists between the presence of 500G/540C in male and positive family history cancer OR=2.76 (95% CI=1.01-7.52, $p<0.05$). These data are very important because variants in the 3'UTR region have been correlated with a shorter disease-free survival (30). Variants in CDKN2A 3'UTR are related with changes in p16 mRNA metabolism (transport, stability and translation) and cell-cycle progression, cell activity, and development of melanoma (26, 29).

Conclusion

According to our results, frequencies in CDKN2A variants show a similar distribution of CDKN2A 3'UTR variants in cases and control groups. No variants were found in either group for CDK4. Additionally, this study indicates that in the Colombian population the presence of haplotype 500G/540C in males is associated with an increased risk of developing melanoma, especially in subjects with a family history of cancer. These results show that the Colombian population can carry other genetic variants related to the development of melanoma. Future studies should replicate these findings and involve subjects from other geographic regions of Colombia.

Conflicts of Interest

The Authors declare no conflicts of interest.

Authors' Contributions

Conceptualization: L.D.G.C; methodology: D.T.P and L.D.G.C; software: D.T.P and S.R.G.Q; validation: J.N and L.D.G.C; formal analysis: D.T.P; investigation: D.T.P and L.D.G.C; data curation: S.R.G.Q; Visualization: D.T.P and L.D.G.C; writing–original draft preparation: D.T.P; writing–review and editing: L.D.G.C, J.N and S.R.G.Q; supervision: L.D.G.C; project administration: L.D.G.C; funding acquisition: L.D.G.C. All authors have read, discussed the results, and contributed to the final manuscript.

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