

## Association between *VDR* Gene Polymorphisms and Melanoma Susceptibility in a Colombian Population

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### Abstract

**Background:** The vitamin D receptor (VDR) is responsible for mediating the effects of vitamin D through regulation of other gene transcriptions. There are several polymorphisms that alter the gene expression or the function of this protein. We aimed to analyze the association between two SNPs of VDR gene and melanoma cancer in Colombian patients. **Methods:** We included 120 healthy individual as controls and 120 melanoma cancer patients as cases. Patients in both groups were matched in terms of gender and age. The genotyping of rs731236 and rs2228570 polymorphisms was performed using PCR-RFLP. The SNPStats program was used to carry out the statistical analysis through a logistic regression model. **Results:** Under dominant model, we found that rs2228570 polymorphism was associated with melanoma cancer risk (C/C vs C/T-T/T, OR: 5.10, 95% CI: 2.85-9.14), whereas rs731236 polymorphism was associated with a protective effect against this cancer (T/T vs T/C, OR: 0.27, 95% CI: 0.14-0.53). **Conclusion:** Our results suggested that both polymorphisms were involved in the development of melanoma cancer, increasing or decreasing this risk.

**Keywords:** Cancer- *FokI* and *TaqI* genes- PCR-RFLPs- rs2228570- rs731236

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### Introduction

Melanoma is a type of skin cancer consisting of fast-growing melanocyte cells. Although melanoma is less common than other types of non-melanoma tumors, such as basal cell carcinoma (BCC) and squamous cell carcinoma (SCC), this type of malignancy is more aggressive. In other words, melanoma spreads to other organs rapidly (Abbas et al., 2014). According to GLOBOCAN 2020 statistics, the worldwide incidence of melanoma was 239,758 cases, and its mortality was about 35,563. In Colombia, its incidence was 1,323 people and its mortality was 317 (Sung et al., 2021). The principal risk factors associated with melanoma development the risk of developing this pathology are exposure to ultraviolet radiation from sunshine and genetic alterations (Rastrelli et al., 2014; Kozovska et al., 2016). Several studies found an association between the risk of melanoma development and different genes, and such as CDKN2A, CDK4, BAP1, POT1, and TERT (Hayward, 2003; Read et al., 2016; Ribero et al., 2016; Nassar and Tan, 2020).

Mutations and polymorphisms in the vitamin D receptor gene (VDR) were highly correlated with melanoma and other types of cancer. VDR is a transcription factor that regulates the expression of several genes through

interaction with a region in some genes named vitamin D responsive element (VDRE) (Shaffer and Gewirth, 2004; Qin and Wang, 2019). VDR regulates different targets involved in many different processes within the cells. Cellular proliferation and angiogenesis processes are inhibited, while apoptosis and cell differentiation are promoted by VDR. Different studies showed the key role of VDR in cancer development, including melanoma (Dou et al., 2016; Slominski et al., 2017; de La Puente-Yague et al., 2018; Trump and Aragon-Ching, 2018; Wu et al., 2019). It was demonstrated that polymorphisms in this gene decreased the protein functionality and its anticarcinogenic properties (Rai et al., 2017; Birke et al., 2020; Matini et al., 2020). Among the polymorphisms of VDR gene, *FokI*, *BsmI*, *TaqI*, *ApaI*, and *Cdx2* are more frequent. These variants lead to instability of its mRNA and induce other isoforms or conformational changes in the structure of the protein (Uitterlinden et al., 2004).

In this investigation, we evaluated *FokI* (rs2228570) and *TaqI* (rs731236) polymorphisms using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. The *FokI* polymorphism is a T/C transition (ATG to ACG), creating an alternative start site. There are two potential translation initiation sites in the exon 2 codon, generating two isoforms of different size

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(Cieslinska et al., 2018).

Taq 1 polymorphism is a T/C nucleotide substitution (ATT to ATC), which leads to a synonymous change at codon of the exon 9 (Cieslinska et al., 2018; Seleit et al., 2020).

These polymorphisms have been evaluated in different types of cancers, such as breast, prostate, skin, and colorectal cancers. For rs2228570 polymorphism, the f allele generates an isoform of VDR less active and effective. Previous investigations suggested that ff (C/C) genotype was associated with increased risk of cancer compared with FF genotype in Caucasian population (Gandini et al., 2009; Raimondi et al., 2009; Tang et al., 2009; Zhao et al., 2014). The Taq 1 (rs731236 polymorphism) TT genotype was associated with increased risk of prostate cancer (Taylor et al., 1996). Another study reported that T/T was associated with the risk of developing lymph node metastasis in breast cancer (Curran et al., 1999). Moreover, the tt/ff genotypes in VDR gene were correlated with a higher risk of triggering melanoma cancer in European people (Hutchinson et al., 2000; Zeljic et al., 2014). To the best of our knowledge, there is no study investigating the relation between these SNPs in VDR gene and melanoma in Latin America population, and specifically in Colombian people. Therefore, this study was performed to analyze the association between two SNPs (rs731236 and rs2228570 polymorphisms) in VDR gene and risk of developing melanoma in Colombian population.

## Materials and Methods

### Participants

Here, we performed a case-control study to analyze the association between two polymorphisms in the VDR gene and melanoma cancer risk. In total, 240 participants were included in this study, 120 were patients with melanoma cancer (case group) and 120 were individuals with no history of cancer (control group). The case group were clinically and histologically diagnosed according to the classification proposed by the American Joint Committee on Cancer. The control group was healthy volunteers matched to melanoma patients by age and other risk factors. Participants in both groups were recruited at the National Cancer Institute (INC) and Mederi hospital in Bogotá, Colombia. A blood sample was collected from all the participants.

### Ethics statement

This study was performed following the recommendations of the declaration of Helsinki and the national normativity (Ministerio de Salud, Resolución N° 008430 de 1993), and it was approved by the Ethic committees at Applied and Environmental Sciences University in Bogotá, Colombia. All the participants provided written informed consent.

### DNA extraction

First, 5 mL to 10 mL of peripheral blood was drawn from each participant and kept in EDTA containing vacutainers. The whole blood was mixed with Histopaque® (Sigma

Aldrich, San Luis, USA) and centrifuged at 1000g for 30 min at 20°C. The buffy coat with the peripheral blood mononuclear cells (PBMCs) was collected, and then genomic DNA was isolated by salting out method (Shaik et al., 2016). Next, DNA integrity was assessed using the agarose gel electrophoresis, and quantification was performed through Nanodrop 2000 (Thermo Scientific, Waltham, MA, USA).

### Genotyping

A PCR followed by restriction fragment length polymorphism (RFLP) technique were employed to genotype rs2228570 and rs731236 polymorphisms in VDR gene. The PCR conditions were as following: 100 ng of DNA, primers (0.15 pmol), dNTP's (0.2 mM), MgCl<sub>2</sub> (1.2 mM), 1U Taq polymerase (Invitrogen, Carlsbad, CA, EUA). The primer sequences were forward 5'-CTG GCA CTG ACT CTG GCT CT-3' and reverse 5'-TGC TTC TCC CTC CCT TT-3' for rs2228570 polymorphism; forward 5'-CAG AGC ATG GAC AGG GAG CAA G-3' and reverse 5'-CGG CAG CGG ATG TAG GTC TGC AG-3' for rs731236 polymorphism, which was based on a previous study (Halsall et al., 2004). PCR program consisted of a cycle at 94°C for 3 min, 30 cycles at 94°C for 30 sec, annealing at 60°C for rs2228570 polymorphism and 62°C for rs731236 polymorphism during 30 sec, 72°C for 30 sec; the last step was at 72°C for 5 min. The PCR products were submitted to enzymatic digestion for 12 hours at 37°C using the Fok I and Taq I enzymes (Thermo Scientific, Waltham, MA, USA). The products of digestion were analyzed using agarose (3%) gel electrophoresis stained with EZ-vision® (Amresco, Solon, OH, USA).

### Statistical analyses

In this study, the observed genotypes were computed and tested for Hardy-Weinberg equilibrium using Pearson goodness-of-fit chi-square test. We performed a logistic regression to detect the association between SNPs and melanoma using the SNPStats program (Sole et al., 2006). The chi-square test (X<sup>2</sup>) was used to detect any possible differences between the groups in the GraphPad Prism 8 program. P value < 0.05 was considered significant and all statistical tests were 2-sided.

## Results

### Descriptive analysis

A total of 240 participants were included in this study. Among these 240 participants, 120 were patients with melanoma (case group) and 120 were healthy subjects (control group). The participants' age average was 59.93 ± 14.9 in the case group and 59.28 ± 13.9 in the control group. We found similar gender distribution in both groups. In controls, 51% were female and 49% were male. In case group, 55% were female and 45% were male. No significant differences were observed for these variables between case and control groups (Table 1).

In this study, we collected additional information on risk, such as hair color, eye color, skin color and type, tanning ability, sunblock use, cancer history in their family, from both groups. The chi-squared test findings revealed

Table 1. Descriptive Statistics of the Analyzed Samples (120 Cases and 120 Controls).

Variable	Cases (%)	Controls (%)	p
Age	59.93	59.28	0.727 <sup>a</sup>
Gender			
Male	54 (45%)	59 (49%)	0.051
Female	66 (55%)	61 (51%)	
Hair Color			
Black or Brown	106 (88%)	106 (88%)	1
Blond(e) or red	14 (12%)	14 (12%)	
Eye Color			
Blue	14 (12%)	12 (10%)	0.678
Other	106 (88%)	108 (90%)	
Skin Color			
Black or brown	64 (53%)	72 (60%)	0.298
Fair	56 (47%)	48 (40%)	
Tanning ability			
Good	42 (35%)	44 (37%)	0.788
Moderate or poor	78 (65%)	76 (63%)	
Cancer History			
No	62 (52%)	70 (58%)	0.299
Yes	58 (48%)	50 (42%)	
Sunblock use +			
Frequently	16 (24.2%)	18 (18.7%)	
Rarely	26 (39.4%)	22 (22.9%)	0.019*
Never	24 (36.4%)	56 (58.3%)	
Sun exposition			
Job	40 (33%)	34 (28%)	0.402
Recreation	80 (67%)	86 (72%)	
Skin type			
Type I	34 (30%)	30 (27%)	
Type II	44 (39%)	46 (41%)	0.511
Type III	20 (18%)	14 (12%)	
Type IV	16 (14%)	22 (20%)	

P-values were calculated by Chi-square test; <sup>a</sup>, Student's t-test; \*Statistically significance; + For this variable, there were missing data for 54 cases and 24 controls.

no significant difference for these variables between cases and controls (Table 1), except for sunblock use. The most common cancers in the patients' family were breast, cervix, gastric, and skin cancers (data not shown).

#### Clinicopathological characteristics of melanoma patients

The following clinicopathological characteristics of melanoma patients were collected: stage, Breslow depth, Clark level, melanoma type, and metastasis status (Table 2). Most of the patients were at stage I (35%). The frequency of tumor thickness (Breslow depth) was as follows: 0-1mm 26 (22%); 1,1-2,0mm 21 (18%); 2,1-4,0mm 38 (32%); > 4,1mm 35 (29%). About 32% of patients were classified as Clark level V. The most common type of melanoma was acral lentiginous melanoma (59%), followed by nodular melanoma (22%), lentigo maligna melanoma (9%), melanoma in situ (7%),

Table 2. Clinicopathological Characteristics of the Melanoma Cancer Patients

Characteristic	n (%)
Stage	
I	35 (29%)
IB	4 (3%)
IIA	23 (19%)
IIB	10 (8%)
IIC	4 (3%)
IIIA	4 (3%)
IIIB	24 (20%)
IIIC	1 (1%)
IV	15 (12%)
Breslow	
0 – 1 mm	26 (22%)
1.1 – 2.0 mm	21 (18%)
2.1 – 4.0 mm	38 (32%)
> 4.1 mm	35 (29%)
Clark	
I	2 (2%)
II	23 (19%)
III	30 (25%)
IV	27 (22%)
V	38 (32%)
Melanoma type	
Lentigo maligna melanoma	11 (9%)
Acral lentiginous melanoma	71 (59%)
Superficial spreading melanoma	4 (3%)
Melanoma <i>in situ</i>	8 (7%)
Nodular melanoma	26 (22%)
Metastasis	
Yes	15 (12.5%)
No	105 (87.5%)

and superficial spreading melanoma (3%). Only 15 patients had metastasis. All data on patients' clinicopathological characteristics are presented in Table 2.

#### Allelic and genotypic frequencies

In the case group, the frequency for alleles T and C of rs731236 SNP were 0.89 and 0.11, respectively. The Hardy Weinberg Equilibrium had a P value of 0.089. In the case group, the allelic frequencies were T= 0.94 and C= 0.06, while T= 0.84 and C = 0.16 in the control group. Only two genotypes were found in these analyzed samples, namely T/T and T/C. The genotypic frequency of T/T was 0.78 and of T/C was 0.22. The genotypic frequency of T/T was 0.88 and of T/C was 0.12 in the case group, while it was 0.68 for T/T and 0.32 for T/C in the control group (Table 3). The allele C from rs2228570 polymorphism was the most frequent allele in the total sample (0.63). The frequency of minor allele was 0.37 for allele A. We found no significant difference from Hardy Weinberg equilibrium test for rs2228570 polymorphism (P: 0.072) in all samples. The genotypic frequencies for rs2228570

Table 3. Allelic and Genotypic Frequencies of *rs731236* and *rs2228570* Polymorphisms in Cases and Controls

	All subjects/ proportion	HWE (P-value)	Cases/ proportion	Controls/ proportion
<i>rs731236</i>				
Allelic frequencies				
T	427/ 0.89		226/ 0.94	201/ 0.84
C	53/ 0.11	0.089	14/ 0.06	39/ 0.16
Genotypic frequencies				
T/T	187/ 0.78		106/ 0.88	81/ 0.68
T/C	53/ 0.22		14/ 0.12	39/ 0.32
<i>rs2228570</i>				
Allelic frequencies				
C	302/ 0.63	0.072	132/ 0.55	170/ 0.71
T	178/ 0.37		108/ 0.45	70/ 0.29
Genotypic frequencies				
C/C	88/ 0.37		23/ 0.19	65/ 0.54
C/T	126/ 0.52		86/ 0.72	40/ 0.33
T/T	26/ 0.11		11/ 0.09	15/ 0.12

HWE, Hardy Weinberg Equilibrium; P-values were calculated by Chi-square test

polymorphism were C/C (0.37), C/T (0.52), and T/T (0.11) for all the patients included in this study (Table 3).

*Association between rs731236 and rs2228570 polymorphisms and melanoma risk*

We analyzed the association between *rs731236* and *rs2228570* polymorphisms and melanoma cancer. For *rs2228570* polymorphism, several genetic models were assessed by means of a logistic regression test. This analysis was not performed for *rs731236* polymorphism since only two genotypes were identified in our samples. We observed a significant association between *rs2228570* polymorphism and melanoma cancer under codominant model (OR=6.3; 95% Confidence interval=3.41-11.62) and dominant model (OR=5.10; 95% confidence interval (2.85-9.14)). The frequency of C/T-T/T genotypes was greater in cases in comparison to controls, suggesting

that this variation increases the risk of melanoma cancer 5.1 times. We also observed a significant association between *rs731236* polymorphism and melanoma cancer (OR=0.27; 95% confidence interval (0.14-0.53), p value of 1e-04). However, for this SNP, the T/C genotype was more frequent in controls, suggesting that this polymorphism was 3.7 times more protective for melanoma cancer (Table 4).

Additionally, we evaluated the association between *rs731236* and *rs2228570* polymorphisms and the clinicopathological characteristics of the melanoma patients. For this purpose, we first grouped the variables as the following: Stage A (I, IB, IIA), Stage B (IIB, IIC, IIIA, IIIB, IIIC, IV), Breslow A ( $\leq 2$  mm) and Breslow B ( $> 2$  mm), and Clark A (I, II, III) and Clark B (IV and V). We only observed a significant difference for Clark variable and the *rs2228570* polymorphism (OR=0.35,

Table 4. Association between *rs731236* and *rs2228570* Polymorphisms and Melanoma Cancer (Adjusted by Age and Gender)

Model	Genotype	Cases	Controls	OR (95% CI)	p
<i>rs731236</i>					
	T/T	106 (88.3%)	81 (67.5%)	1	
---	T/C	14 (11.7%)	39 (32.5%)	0.27 (0.14-0.53)	<b>0.0001</b>
<i>rs2228570</i>					
Codominant	C/C	23 (19.2%)	65 (54.2%)	1	
	C/T	86 (71.7%)	40 (33.3%)	6.3 (3.41-11.62)	<b>&lt;0.0001</b>
	T/T	11 (9.2%)	15 (12.5%)	2.06 (0.82-5.14)	0.117
Dominant	C/C	23 (19.2%)	65 (54.2%)	1	
	T/C-T/T	97 (80.8%)	55 (45.8%)	5.10 (2.85-9.14)	<b>&lt;0.0001</b>
Recessive	C/C-T/C	109 (90.8%)	105 (87.5%)	1	
	T/T	11 (9.2%)	15 (12.5%)	0.70 (0.30-1.59)	0.392
Overdominant	C/C-T/T	34 (28.3%)	80 (66.7%)	1	
	T/C	86 (71.7%)	40 (33.3%)	5.26 (3.01-9.18)	<b>&lt;0.0001</b>

P-value was calculated by Chi-Square test; p<0.05 was considered significant and are depicted in bold

Table 5. Association between *rs731236* and *rs2228570* Polymorphisms of VDR Gene and Clinicopathological Characteristics of Melanoma Cancer Patients

	<i>rs731236</i>				<i>rs2228570</i>						
	TT (%)	TC (%)	OR (95% CI)	p	CC (%)	TC (%)	TT (%)	TC + TT (%)	†OR (95% CI)	p	
Stage											
I – IIA	53 (85.5)	9 (14.5)	0.56 (0.17-1.77)	0.319	11 (17.7)	45 (72.6)	6 (9.7)	51 (82.3)	0.83 (0.33-2.05)	0.682	
IIB – IV	53 (91.4)	5 (8.6)			12 (20.7)	41 (70.7)	5 (8.6)	46 (79.3)			
Breslow											
≤ 2 mm	43 (91.5)	4 (8.5)	1.71 (0.50-5.80)	0.391	9 (19.1)	32 (68.1)	6 (12.8)	38 (80.9)	0.99 (0.39-2.53)	0.997	
> 2 mm	63 (86.3)	10 (13.7)			14 (19.2)	54 (74)	5 (6.8)	59 (80.8)			
Clark											
I + II + III	49 (89.1)	6 (10.9)	1.15 (0.37-3.53)	0.238	10 (18.2)	41 (74.5)	4 (7.3)	45 (81.8)	0.35 (0.13-0.95)	<b>0.031</b>	
IV + V	57 (87.7)	8 (12.3)			13 (20.0)	45 (69.2)	7 (10.8)	52 (80.0)			
Metastasis											
Negative	93 (88.6)	12 (11.4)	1.19 (0.24-5.94)	0.83	18 (17.1)	78 (74.3)	9 (8.6)	87 (82.9)	0.41 (0.13-1.36)	0.145	
Positive	13 (86.7)	2 (13.3)			5 (33.3)	8 (53.3)	2 (13.3)	10 (66.7)			

Logistic regression analysis was adjusted for age and gender; OR, Odds Ratio; CI, Confidence Interval; †, dominant inheritance model; p<0.05 was considered significant and are depicted in bold.

95% confidence interval (0.13-0.95), p-value of 0.031). T/C-T/T genotypes were more frequent in patients with a Clark classification of I, II, III in comparison to those with that of IV and V.

## Discussion

Cancer affects many people in the worldwide. The study of the genetic basis of this disease continues to be of great importance. Numerous gene variations are associated with the risk or the protection for this pathology (Aristizabal-Pachon et al., 2015a; Aristizabal-Pachon et al., 2015b; Aristizabal-Pachon and Takahashi, 2016; Aristizabal-Pachon and Castillo, 2017; Rangel et al., 2020), including VDR gene (Birke et al., 2020). VDR has several SNPs, which may affect the amino acid sequence of the protein. Some variations affect the gene expression and consequently alter the RNA stability, such as *rs731236*. However, other SNPs trigger alterations on protein structure like *rs2228570* polymorphism (Jakubowska-Pietkiewicz et al., 2012). In the current study, for the first time, we studied these polymorphisms of VDR gene in a Colombian sample and found significant associations between these polymorphisms and the risk of or protection against melanoma cancer.

Here, we observed that carriers of T/C-T/T genotypes induce more risk to develop melanoma skin cancer in comparison to CC carriers of *rs2228570* polymorphism. T allele had minor frequency in our study (0.37), which is commonly denoted as f. This variation induces a less effective protein (Whitfield et al., 2001), which can alter the control of expression of several genes with anticarcinogenic action (Rai et al., 2017). This can explain the higher susceptibility of developing melanoma cancer in T allele carriers. The association observed in the present study was similar to the findings reported by a meta-analysis in 2020 (Birke et al., 2020). In the aforementioned study, the authors included 11 studies with a total of 4,506 melanoma cases and 4,409 healthy controls. They reported that carriers of the rarer allele

had 22% more risk to develop a malignant melanoma. In contrast to our findings, the authors did not find any significant results for *rs731236* in nine included studies (4,319 melanoma cases and 3,630 healthy controls) (Birke et al., 2020). The T/C genotype was shown to have a protective effect for melanoma cancer risk (OR=0.27 (0.14-0.53)), which is in line with results presented by Li et al., (2008) in Non-Hispanic Whites in Texas. In their study, the authors found that this SNP was associated with a significantly reduced melanoma cancer risk. It should be noted that *rs731236* polymorphism was also associated with a protective effect for breast cancer according to a previous study (Randerson-Moor et al., 2009).

In this study, we also analyzed the association between these polymorphisms and clinicopathological characteristics of the melanoma patients in order to find out whether they were associated with progression or with the stage of the disease or not. We only observed a significant association between *rs2228570* polymorphism and Clark level (Table 5), which can provide information about the invasion level (Susok et al., 2021). Although the T/T-T/C genotypes were associated with an increased risk for developing melanoma cancer, the analysis of pathological characteristics, as Clark level, revealed an association with lower levels (I, II, III), suggesting that *rs2228570* polymorphism may be involved with a less degree of invasion though the role of this polymorphism in cancer invasion is not clear yet. Nevertheless, a study evaluated the expression of VDR protein in melanoma cells and detected an association with Clark levels reporting the presence of negative cytoplasmic VDR in higher levels (Randerson-Moor et al., 2009).

In conclusion, we found an association between two polymorphisms of VDR gene and melanoma cancer in a Colombian sample. However, the association was in two directions, that is *rs2228570* polymorphism was associated with the risk of developing this cancer, while *rs731236* polymorphism has protective effects against melanoma cancer. Further studies recruiting more samples are necessary to confirm our findings. Moreover,

studies analyzing interactions between other genes and environmental factors could provide additional insights into the role of these polymorphisms in the development of melanoma cancer. Additionally, there is a need for genetic studies on the Latin-American population since most studies were performed in the American and European populations.

### Author Contribution Statement

AFAP and JGS conceived and designed this study. AFAP, DS and AP collected samples and performed the PCR-RFLPs. AFAP and YGG performed statistical analysis and data interpretation. AFPA, YGG and AYG collected clinical data and wrote the paper. All authors read and approved the final manuscript.

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#### Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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