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Melanoma: clinic-pathological and molecular analyses in patients from Ibague, Colombia

Melanoma: análisis clínico-patológico y molecular en pacientes de la ciudad de Ibagué, Colombia

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ABSTRACT

This study aims to establish the clinicopathological characteristics of patients with melanoma and their association with BRAF gene mutations. Pathology reports and paraffin-embedded tumor samples from 47 ´¡ women and 30 men with melanoma and an average age of diagnosis of 60 were examined at the Hospital Federico Lleras Acosta in Ibague, between 2010 and 2016. The presence of V600E mutation at BRAF exon 15 was analyzed in these tumoral samples by Sanger sequencing and visual inspection of electropherograms. Clinicopathological variables were also studied using X2, t-Student and the Kaplan Meier index. Most of the lesions were located in the lower limbs (46.6 %). The most frequent subtype was acral lentiginous melanoma (41.8 %). Most lesions were of poor prognosis: Breslow depth greater than 4.1 mm (52.7 %), ulceration (61.4 %) and medium or high mitotic rate (> 30 %). V600E mutation was identified in five patients with large, deep and ulcerated tumors, four of whom had less than four years of survival. In conclusion, melanoma is highly frequent in women, V600E BRAF mutation is present in patients with advanced disease (high Breslow index), and the five-year survival rate is less than 40 %.

Keywords: Melanoma; mutations; BRAF; skin neoplasms.

RESUMEN

El objetivo de este estudio fue establecer las características clínico-patológicas de los pacientes con melanoma y su asociación con mutaciones del gen BRAF. Los informes de patología y las muestras de tumores incluidos en parafina de 47 mujeres y 30 hombres con melanoma, con una edad promedio de diagnóstico de 60 años, se revisaron en el Hospital Federico Lleras Acosta de Ibagué, entre 2010 y 2016. En estos tejidos tumorales se detectó la presencia de la mutación V600E del exón 15 del gen BRAF, mediante secuenciación por el método de Sanger e inspección visual del electroferograma. Además, se estudiaron las variables clínico-patológicas con X2, t-Student e índice Kaplan Meier. La mayoría de las lesiones estaban localizadas en las extremidades inferiores (46,6 %). El subtipo más frecuente fue el melanoma lentiginoso acral (41,8 %). La mayoría eran de mal pronóstico: profundidad superior a 4,1 mm (52,7 %), ulceración (61,4 %) y tasa mitótica media o alta (> 30 %). La mutación V600E se identificó en cinco pacientes con tumores grandes, profundos y ulcerados, cuatro de ellos tuvieron una supervivencia menor de cuatro años. En conclusión, hubo mayor frecuencia de melanoma en mujeres, la mutación V600E BRAF se presentó en pacientes con enfermedad avanzada y la probabilidad de supervivencia a cinco años fue inferior al 40 %.

Palabras clave: melanoma; mutaciones; BRAF; neoplasias de la piel

INTRODUCTION

Melanoma is a tumor of aggressive behavior that develops in melanocytic cells, mainly the skin, although it may also appear in the eye and various mucosal surfaces[1,2]. It is a rare and heterogeneous form of cancer with a complex multigenic etiology[3,4]. Its high degree of malignancy and high tendency to metastasize result in an unfavorable prognosis, since the overall five-year survival rate for patients with more than three metastases to lymph nodes or distant organs is less than 16 %, representing 90 % of deaths associated with skin tumors[5-7]. Interestingly, in Colombian studies, the overall melanoma-specific five-year survival rate is substantially lower than that reported by countries with higher incidence[8].

According to its clinical and histopathological characteristics, melanoma is classified into four subtypes: superficial spreading melanoma, generally flat and irregular in shape and color; nodular melanoma, of a brown-black color, characterized by an aggressive vertical growth phase; lentigo maligna melanoma, which appears mainly in the elderly on skin exposed to the sun (face, neck and arms), described as a lentiginous proliferation of atypical melanocytes; and finally, the rare acral lentiginous melanoma, which occurs mainly on the palms of the hands and the soles of the feet and, during the initial intraepidermal phase, has irregular pigmentation and may later exhibit a nodular formation with invasive growth[4,9,10].

In 2017, the American Cancer Society reported about 87,100 new cases of melanoma with a mortality of 9,730 in the United States[5,11]. In general, the incidence rate has been increasing around the world, especially in people with white skin who have higher exposure to the sun[9,12]. The incidence rate of new cases of melanoma per 100,000 inhabitants is 10-25 in Europe, 20-30 in the United States, and 50-60 in Australia[1,11,13-15].

Since Colombia does not have a unique cancer registry system and only some reports on its estimated incidence are known[16], it is difficult to establish general data on the affected population. In 2012, GLOBOCAN reported an incidence of melanoma of 2.3 and 1.9 for men and women, respectively, and a mortality rate of 0.9 %, evidencing a decrease compared to 1.3 % reported in 2010 by the National Institute of Cancerology, which can be attributed to early diagnosis and prevention campaigns[16].

In this sense, it is essential to implement methods that pinpoint the prognostic factors of the disease, including the Breslow classification, which helps determine the extent of melanoma and is considered one of the most important clinicopathological features when predicting the survival time of patients with melanoma[5]. According to Classic Pathology, the chances of a patient being treated and cured in early stages of the disease are high, compared with those of a person in an advanced state that has low probability of survival. The authors conclude that patients with tumors larger than two millimeters thick are at risk of developing loco-regional cutaneous metastases[17].

The progress of molecular studies has shown that solar ultraviolet (UV) radiation is an important environmental risk factor for the development of melanoma[3,13,15,18]. It has been shown that the risk varies depending on geographical location, type of skin, hair color, number of nevi and family history of melanoma[3,18,19] .

The growing incidence of melanoma worldwide has stimulated an increase in research aimed at identifying the genetic, environmental and phenotypic factors that contribute to its pathogenicity[3,12]. Recent studies have shown that between 40 and 60 % of cases show somatic mutations in the BRAF gene, with the V600E mutation being the most frequent (50 %) in patients with metastatic melanoma[20] and usually has a spindle cell morphology[21].

This study analyzes the clinicopathological factors in patients with melanoma, as well as the characteristics of the tumor and survival using the Kaplan Meier index. In addition, BRAF exon 15 was sequenced to establish the presence of somatic mutations in the DNA obtained from paraffin-embedded tumor tissues.

MATERIALS AND METHODS

Population, sample and type of study

This study is part of the program "Genetic Analysis of Human Diseases" developed by the Cytogenetics, Phylogeny and Evolution of Populations research group of the Universidad del Tolima since 2005, which was approved by the University's Bioethics Committee and the Hospital Federico Lleras Acosta (HFLLA). We reviewed 77 pathologies of patients diagnosed with melanoma during the 2010-2016 period. Records were anonymized and epidemiological (age of diagnosis, gender, and year of death), clinical (location and histological type) and pathological (ulceration, Breslow index, and mitotic rate) variables analyzed.

Instruments and procedures

Tissue embedded in paraffin was obtained from 56 patients residing in Ibague, Tolima. A pathologist (MEB) demarcated tumor regions with > 80 % of tumor cells on H&E slides and, from them, total DNA extraction was performed by the silica microcolumn extraction protocol (QIAGEN KIT, Dneasy - Blood & Tissue Kit, Cat. N° 69504), following the manufacturer's recommendations.

Amplification of BRAF exon 15 was performed using conventional PCR with forward (5'-AGAAATTAGATCTCTTACCTAAACT-3') and reverse (5'-TTACCATCCACAAAATGGA-3') primers. As a positive control, DNA extracted from a positive thyroid cancer patient was used for the BRAF V600E mutation and, as a negative control, ultra-pure water was used instead of DNA. Each mixture was brought to a final volume of 25 µl. The thermal profile consisted of an initial denaturation at 95 °C for 5 minutes, followed by 35 cycles of denaturation, alignment and extension at 95 °C (1 minute), 55 °C (1 minute) and 72 °C (1 minute), and a final extension at 71 °C for 10 minutes.

Amplified products were visualized by agarose gel electrophoresis and sequenced by Sanger at Macrogen (Korea). Sequences were aligned using BLAT in the UCSC Genome Browser (https://genome.ucsc.edu/cgi-bin/hgBlat?command=start) with human genome version GRCh38/hg38. Mutation status in BRAF (V600E) was visually inspected in electropherograms with 4Peaks v. 1.7 by two experienced independent researchers (CJP, MEB). Mutation calling concordance was 100 %.

Statistical analysis

For the statistical analysis, VassarStats algorithms were used to calculate the X2 and t-Student tests and R version 1.68 for the Kaplan Meier test. Variables were described in frequencies or means and standard deviations.

Data were compared with information from global (The Cancer Genome Atlas Program -TCGA) and local databases, for clinic and histological characteristics, as Breslow index, including ulceration and histological type, so as reported mutations and survival rates.

Ethical considerations

To guarantee compliance with the principles and ethical standards of the Declaration of Helsinki of 1975, as amended, and Resolution 8430 of 1993 by the Colombian Ministry of Health, and after authorization of the research program, a search was made in the database and the clinical history of patients, and data, as well as paraffin blocks and histology slides, were tabulated in a randomized manner and totally anonymized.

RESULTS

Clinicopathological data are summarized in Table 1. A total of 77 patients (47 women and 30 men) from the city of Ibague with a diagnosis of melanoma confirmed by histopathology were included. No significant differences were found in the average age of diagnosis between genders ($t = 1.03$, $df = 75$, $P = 0.306$), being 59.54 ± 6.8 years for men and 60.12 ± 5.12 years for women. It is highlighted that 57 % of people were diagnosed before age 60. Annual distribution of cases was 17 in 2010, 19 in 2011, 18 in 2012, 13 in 2013, 3 in 2014, 5 in 2015, and 2 in 2016.

The anatomical locations were, in order of importance, lower limbs (48.8 % in women and 43.3 % in men), head and neck, and upper limbs (Table 1). In 41.8 % of the cases there was acral melanoma (17 women and 11 men), affecting the soles of the feet, nails and palms, followed by atypical melanocytic lesions with 22.4 % (Table 1).

The depth of tumors, measured according to the Breslow index, showed that 52.7 % (29 of 55 cases) had a lesion deeper than 4 mm and in 47.3 % (26 people) it was less than or equal to 4 mm; 22 pathology reports were missing the Breslow index (Table 1 and Figure 1).

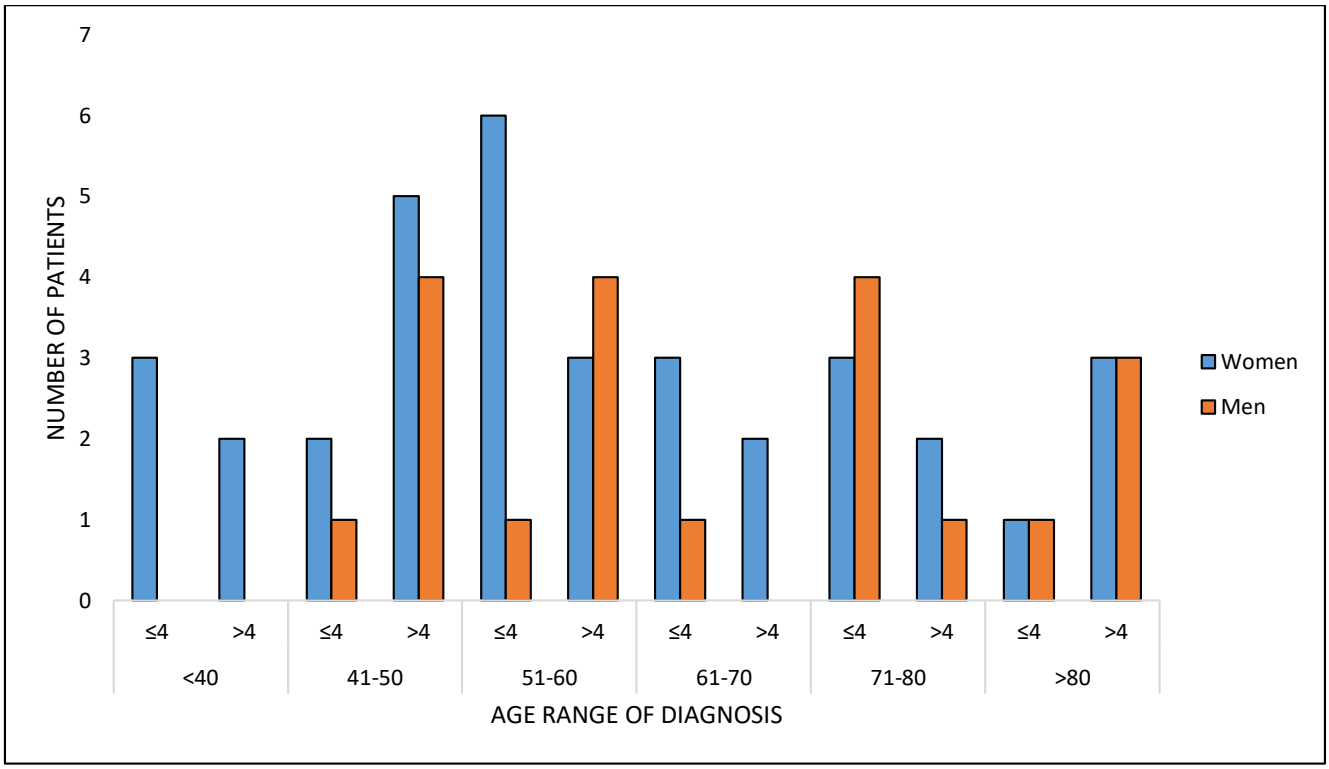

Figure 1. Depth of lesion (Breslow) according to gender and age of diagnosis.

Ulceration occurred in 61.4 % (35 people) and was more evident at a higher age of diagnosis. It is noteworthy that this was the only variable with significant differences between genders (p = 0.042), being more frequent in men (81 %) than in women (50 %) (Table 1 and Figure 2).

Table 1. Clinicopathological Characteristics of Patients Included in the Sample.

Variable		Women n (%)	Men n (%)	Total n (%)	P Value
Age	≤ 60	29 (61.7)	15 (50)	44 (57.1)	0.4386
	> 60	18 (38.3)	15 (50)	33 (42.9)	
	Average	60.1	59.5	59.9	
Biopsy or resection	Biopsy	23 (52.3)	11 (44)	34 (49.3)	0.6801
	Resection	21 (47.7)	14 (56)	35 (50.7)	
	Not reported	3	5	8	
Anatomical location	Head and neck	9 (20.9)	4 (13.3)	13 (17.8)	0.292
	Lower limbs	21 (48.8)	13 (43.3)	34 (46.6)	
	Upper limbs	6 (14)	4 (13.3)	10 (13.7)	
	Trunk	5 (11.6)	4 (13.3)	9 (12.3)	
	Other	2 (4.7)	5 (16.7)	7 (9.6)	
	Not reported	4		4	
Histological type	Acral lentiginous	17 (40.5)	11 (44)	28 (41.8)	0.2977
	Lentigo maligna	7 (16.7)	1 (4)	8 (11.9)	
	Atypical melanocytic lesion	7 (16.7)	8 (32)	15 (22.4)	
	Superficial spreading melanoma	3 (7.1)	0	3 (4.5)	
	Nodular	8 (19)	5 (20)	13 (19.4)	
	Not reported	5	5	10	
Diameter	≤ 20	23 (50)	13 (48.1)	36 (49.3)	0.9203
	> 20	23 (50)	14 (51.9)	37 (50.7)	
	Not reported	1	3	4	
Depth (Breslow)	≤ 4 mm	18 (51.4)	8 (40)	26 (47.3)	0.5902
	> 4 mm	17 (48.6)	12 (60)	29 (52.7)	
	Not reported	12	10	22	
Ulceration	No	18 (50)	4 (19)	22 (38.6)	0.0419
	Yes	18 (50)	17 (81)	35 (61.4)	
	Not reported	11	9	20	
Mitotic rate	Low	12 (34.3)	7 (29.2)	19 (32.2)	0.2516
	Moderate	15 (42.9)	6 (25)	21 (35.6)	
	High	8 (22.9)	11 (45.8)	19 (32.2)	
	Not reported	12	6	18	

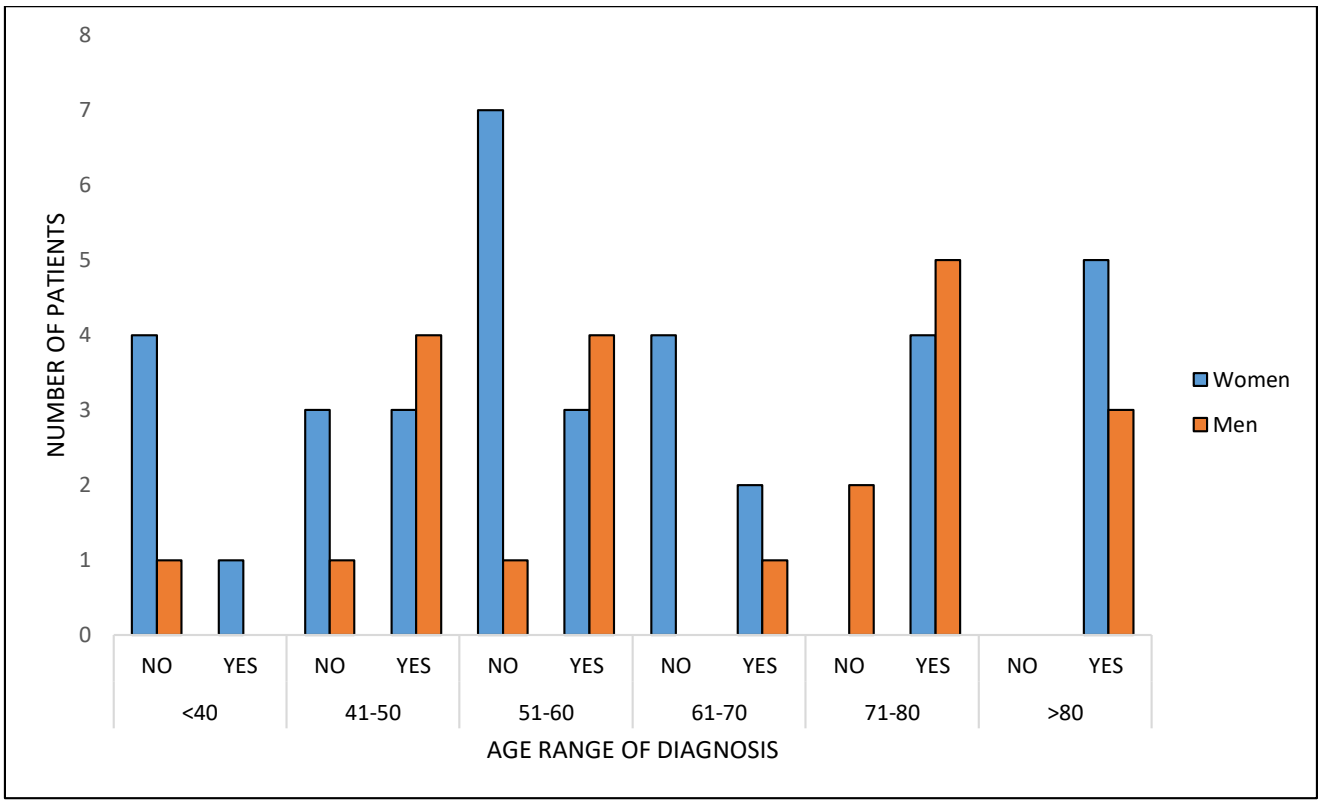

Figure 2. Presence of ulceration according to gender and age of diagnosis.

The mitotic rate did not show significant differences between genders and age of diagnosis (Table 1 and Figure 3), although it tends to be higher in patients with nodular melanoma, atypical melanocytic lesion, and acral lentiginous melanoma (Figure. 4).

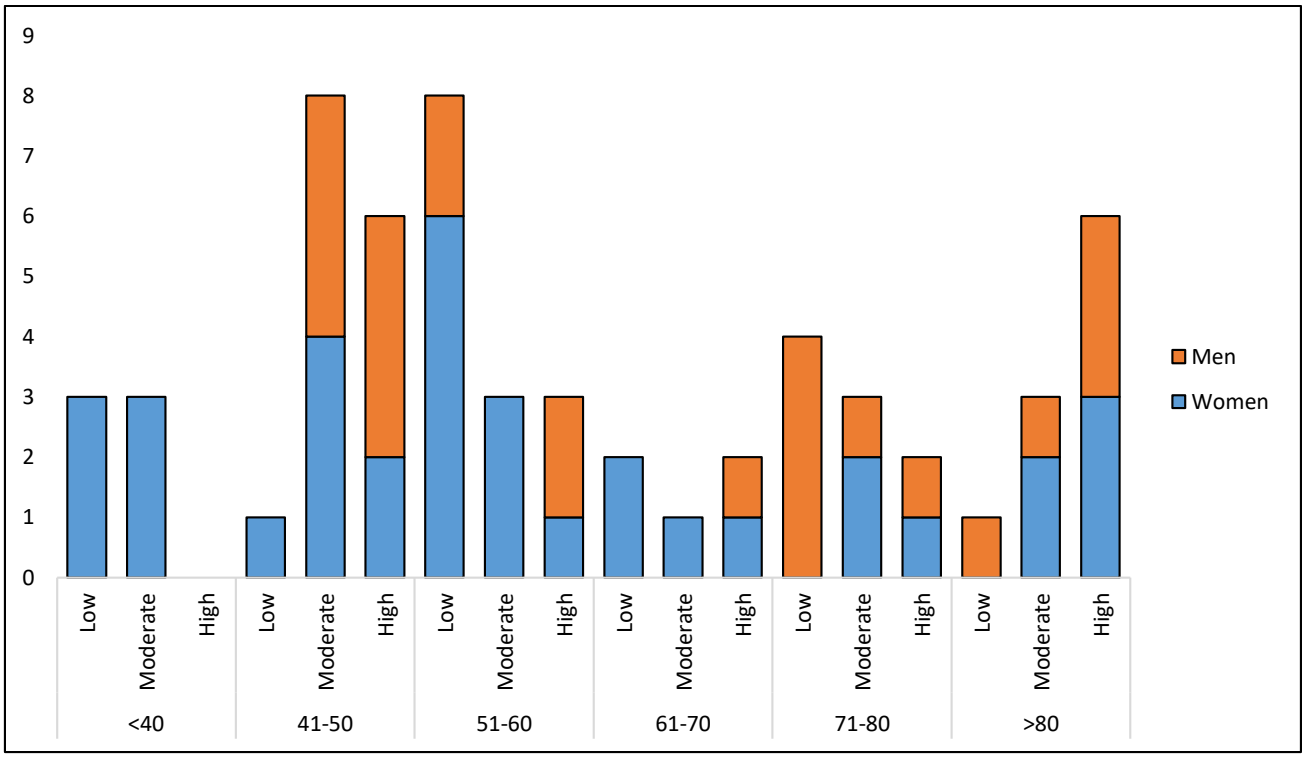

Figure 3. Mitotic rate according to gender and age of diagnosis.

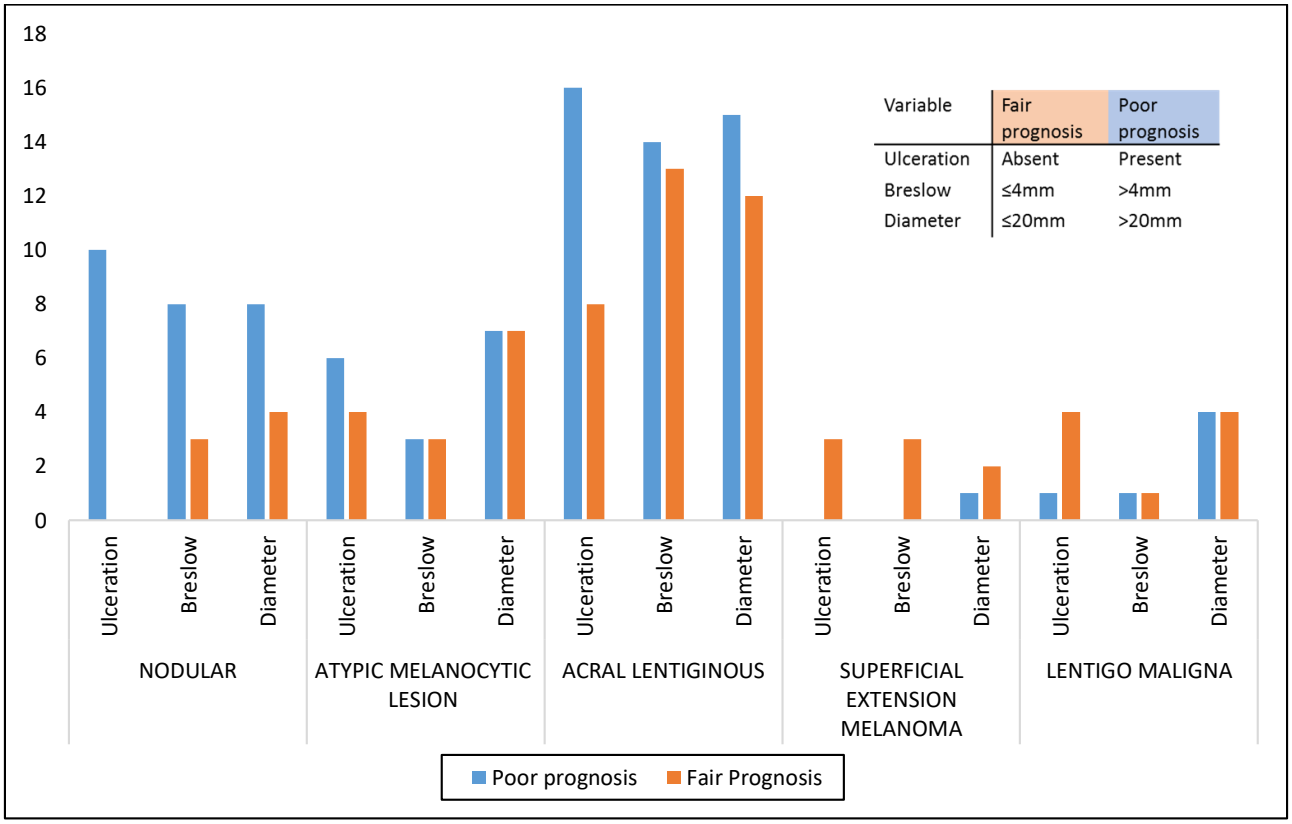

Figure 4. Characteristics of the tumor by histological type.

In relation to survival, 46 patients (59 %) died. From the year of diagnosis to death, a Kaplan Meier test was performed, which revealed that the survival of patients drastically decreased to less than 50 % from the second year after diagnosis and the probability of surviving four years or more was lower than 40 % (Figure 5).

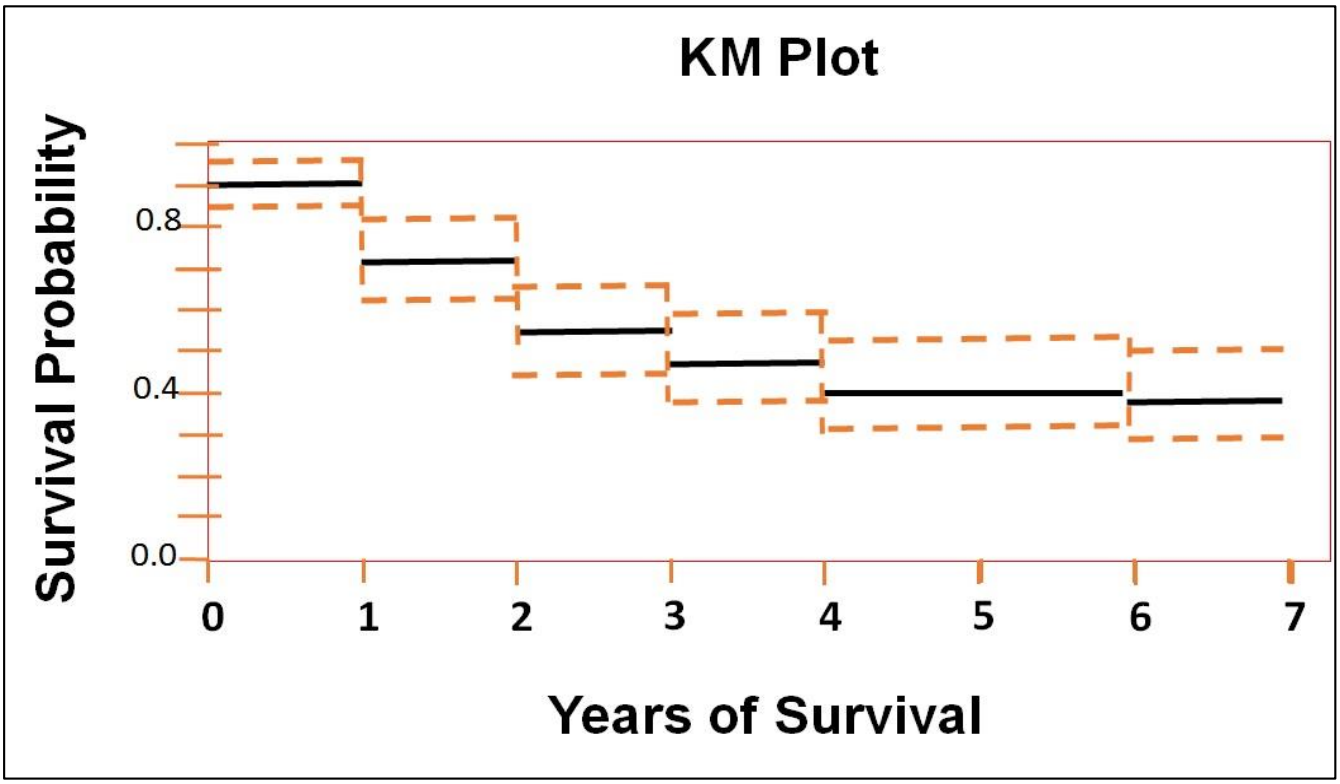

Figure 5. Kaplan Meier Survival Test.

From the 77 pathologies, 56 paraffin-embedded tumor tissues were donated but only 41 tumors had regions with > 80 % of tumor cells on H&E slides for DNA extraction. However, due to the variation of the fixation methods used and the diverse types of paraffin embedding, the DNA extracted had low concentration and was highly fragmented. BRAF exon 15 was efficiently amplified and sequenced in 24 of the cases, obtaining electropherograms with clearly differentiable peaks in 11 of them.

Difficulties in DNA extraction, amplification and sequencing from paraffin-embedded tumors, and specifically from melanomas, has been reported previously. This can be explained by DNA fragmentation and degradation due to treatment with alcohols and formaldehyde, which is carried out for fixation in paraffin blocks, and exposure to high temperatures when inadequate paraffin is used for the process. In addition, the high concentration of melanin present in the skin, and even more so in melanomas, is a possible inhibitor of PCR, since melanin can bind and inactivate at least a portion of the DNA to be amplified, limiting the binding of DNA polymerase and affecting both the amplification and speed of extension thereof[22,23].

Five of the 11 correctly sequenced samples were V600E positives (Table 2). Four of these cases were men, and three of them died from the disease (in developing countries, patient follow-up is poor, so we have not exact data about the disease stage at the time of death) before reaching four years after diagnosis. The survivor was diagnosed at 22 years of age, with melanoma having epidural metastasis in 2012. The only woman with the mutation was diagnosed at 49 years with a 65 mm tumor (depth of 50 mm) and a survival of less than four years. All cases positive for V600E showed ulceration, except in a patient older than 80 years with an atypical melanocytic lesion.

Table 2. Clinicopathological Data of Ibague Population vs. TCGA Data.

CLINICOPATHOLOGICAL DATA	NUMBER OF PATIENTS		P value
A. Patient Clinical Information	Colombia n = 77	TGCA n = 333	
Age (at diagnosis), in years			
Mean	59.9	56.5	0.136631
Median	57	57.0	
Range	10 to 98	15 to 90	
Gender			
Male n (%)	30 (39.0)	182 (62.0)	0.00042
Female n (%)	47 (61.0)	111 (38.0)	
B. Features of Initial Melanoma Diagnosis			
Known primary melanoma tumor			
Yes	56 (100)	262 (90.0)	0.02752
No	0	29 (10.0)	
Location of primary melanoma			
Head/Neck	13 (16.9)	25 (8.7)	0.059
Trunk	9 (11.7)	106 (36.8)	0.00004
Extremities	44 (57.1)	120 (41.6)	0.021
Other	7 (9.1)	8 (2.8)	0.031
Unknown	4 (5.2)	29 (10.1)	0.270
Primary tumor ulceration at diagnosis			
Yes	35 (61.4)	99 (49.5)	0.638
No	22 (38.6)	101 (50.5)	0.001
Not reported	20	.	.
C. Mutation Status			
BRAF status	n = 11	n = 289	
BRAF + wild type	6 (54.5)	154 (53.3)	1.0
BRAF + V600E	5 (45.5)	122 (42.2)	

DISCUSSION

Melanoma is a public health problem; its incidence and mortality have increased mainly in European countries, the United States and Australia[1,9,15]. In Latin American countries such as Colombia, there are few reports published for this pathology[16,24].

This study reveals that it is more frequently diagnosed in women than in men, according to pathological anatomy reports. However, this difference seems to decrease with age, which agrees with studies conducted in the United States, England and some Nordic countries, as well as those reported by the National Cancer Institute of Colombia[15,16].

Based on some reports, women between 15 and 49 years of age have the highest melanoma rates, but men over 50 are more likely to develop the disease. It is possible that in the case of women, they may be more intermittently exposed to the sun and UV rays;

there are multiple studies showing that BRAF mutations are most commonly developed in this type of exposure[25]. In the case of older men, they do not usually use sunscreen, hats or clothing that protect them from UV exposure; therefore, they are exposed for longer periods of time throughout their life[26,27]. Other studies have indicated that differences in the physiology of men and women play an important role in the development of melanoma; the skin of the man is thicker, secretes more sebum and has less subcutaneous fat, while that of women tends to repair more quickly the damage induced by UV radiation, as revealed by a Dutch study whose data show that the doses to elicit an immunosuppression response were three times lower in men than in women[15].

The results of this study show that the most frequent histological subtype corresponds to lentiginous acral melanoma (41.8 %), congruent with that reported by the INC for Colombia[16], De Vries et al.[24] in 2017 for Cali and Sheen et al. in 2017 for Asian populations[28]. In contrast, in Caucasian populations there seems to be higher frequency of the superficial spreading melanoma[17] subtype (63.2 %), followed by the nodular subtype (50.4 %)[16,24,28,29]. Differences between frequencies of melanoma subtypes in Colombia, with respect to Caucasian populations, can be attributed to the ethnic origin of each population and miscegenation.

Regarding the anatomical location of lesions, both men and women exhibit melanoma more frequently in the lower limbs, head and neck, respectively. It has been reported that in British population men seem to develop detected melanoma in the trunk and women in the lower limbs[18].

The Breslow index is regularly used as a predictor of survival of patients diagnosed with melanoma and has been shown to be one of the most important clinicopathological characteristics[5]. According to this index, patients diagnosed with thin melanomas (less than 2 mm thick) in early stages have a lower risk of loco-regional cutaneous metastases and, in general, a good prognosis[5,11]. Meanwhile, the prognosis of cure for patients with melanoma of more than 2.1 mm thick is limited and the probability of five-year survival after diagnosis is unfavorable[5]. In this study, greater frequency was observed in the Breslow thickness (> 4.1 mm) and it can be deduced that these patients are associated with poor prognosis and higher mortality[1,13.] This may be due to late consultation and difficulties in accessing the health system in our country. Patients diagnosed with malignant melanoma and a tumor depth of more than 2 mm are at high risk of developing metastases[5.] It has been reported that in elderly patients, this characteristic is linked to cerebral metastasis and lower survival[20,30].

Ulceration is another adverse prognostic factor related to the tumor, mainly if it is deeper than 3 mm. This results in a survival rate of approximately 50 % at 10 years in relation to a 78 % if there is no presence of ulceration[27]. The depth or increasing Breslow index is usually accompanied by a high probability of coming into contact with the lymphatic vessels and is evidence of rapid tumor growth[5].

Of the 11 patients with sequenced BRAF exon 15 in the DNA of paraffin-embedded tissue, five had the V600E mutation of the BRAF gene. These patients had advanced disease and died early. In addition, one of the patients was diagnosed before age 30, which is consistent with that reported by Thomas et al., who describe a significant association between the presence of mutations in the BRAF gene and the diagnosis of superficial spreading melanoma at an early age, located in the trunk and extremities and in an advanced tumor stage[31]. Although these results correspond only to ~ 20 % of the total sample, they coincide with the reports that approximately half of the patients with melanoma have some mutation in the BRAF gene, with the V600E mutation being the most frequent in younger patients (under 55 years of age)[32], both at the time of diagnosis of primary melanoma and in the diagnosis of metastasis[20]. Jakob et al. reported that there is no association between the BRAF mutation and the Breslow depth index, the mitotic rate or the ulceration in melanoma, although the highest frequency of the mutation occurs in melanoma with superficial morphology, followed by nodular, lentigo maligna and lentiginous acral subtypes[33,34]. However, in this analysis (Table 2) the V600E BRAF mutation was identified only in patients with advanced disease, worsening the already poor prognosis by the lack of response to a specific antigen therapy[35].

As already pointed out in this study, it was not possible to carry out gene sequencing in all the

tumors of the sample, mainly due to the conditions in the fixation processes that seem to affect the molecular quality of paraffin-embedded samples. Different conditions such as pH, the nature of the fixative and its concentration can promote cross-linking between nucleic acids and proteins in the material preserved in paraffin and formaldehyde, hindering the extraction of DNA and inhibiting PCR[36,38].

The five-year survival rate in patients diagnosed with malignant melanoma in the sample analyzed is 40 %, lower than that reported by the National Cancer Institute of Colombia (54 %)[39]; in other Latin American and Asian studies, values are very close[40-42]. The probability of survival in Colombia is low compared to the United States (82.6 %) and some European countries (France 76.0 % and Germany 82.0 %)[43-45] where incidence is higher. This low probability of survival is because, in Colombia and Latin American countries, the disease is diagnosed in an advanced state, highly increasing the probability of death from 7.4 % in no metastatic state and small thickness, as reported in other countries, to our reported data of 60 %[46,47], possibly due to the precarious access to the health system.

Currently, a limiting factor in melanoma research in the country is the lack of information on incidence and mortality rates. The most complete registry available is that of GLOBOCAN in 2012; however, given that Colombia does not have a single population registry that allows unifying cases of melanoma at the national level, statistics are based on reports from the cities of Medellin and Pasto. Moreover, complete epidemiological data are not recorded, which made it difficult to determine the frequency with which melanoma occurs in the Ibague population. In conclusion, it could be stated that the lack of information coverage indicates that, in works such as those carried out in Cali, the INC and even the present study, the actual magnitude of melanoma may be underestimated[16,24]. This work opens the door to other studies that may generate early detections for this type of skin cancer, reported as the most aggressive, and encourage prevention campaigns that help increase early prognosis and prevent and reduce the development of the pathology.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest.

AUTHORS' CONTRIBUTIONS

First (CJP) and second (APE) authors: DNA extraction, PCR analysis, and result analysis.
Third author (MEB): Study of paraffin-embedded samples, tissue extraction for DNA analysis, result analysis, and manuscript preparation.
Fourth author (AMV): Data sorting, tabulation, result analysis, and manuscript drafting.
Fifth author (CAG): Bioethical approval, obtaining permits and clinical records.
Sixth author (MME): Methodology review and result analysis.

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