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Analysis of the *BRAF* V600E mutation in primary cutaneous melanoma

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ABSTRACT. *BRAF* V600E is the most common mutation in cutaneous melanomas, and has been described in 30-72% of such cases. This mutation results in the substitution of valine for glutamic acid at position 600 of the BRAF protein, which consequently becomes constitutively activated. The present study investigated the *BRAF* V600E mutation frequency and its clinical implications in a group of 77 primary cutaneous melanoma patients treated in a cancer reference center in Brazil. Mutation analysis

was accomplished by polymerase chain reaction, restriction fragment length polymorphism, and automated DNA sequencing. The chi-squared and Fischer exact tests were used for comparative analyses. The *BRAF* V600E mutation was detected in 54/77 (70.1%) melanoma subjects. However, no statistically significant association was found between the presence of the mutation and clinical or prognostic parameters. Our results demonstrated that the *BRAF* V600E mutation is a common event in melanomas, representing an important molecular target for novel therapeutic approaches in such tumors.

Key words: *BRAF* V600E; MAPK; Mutation; Cutaneous melanoma

INTRODUCTION

The prognosis for metastatic melanoma patients is distressing. Up to 95% of stage IV melanoma patients die within five years after diagnosis and a great number die within one year despite standardized treatment (Sullivan and Flaherty, 2011). According to the American Joint Committee on Cancer (AJCC), independent predictive survival factors for melanoma patients include, in order of decreasing significance: tumor thickness (Breslow index), mitotic rate, patient age, ulceration, anatomic site, and gender (Thompson et al., 2011).

In the past few years, scientific knowledge of the molecular composition of melanomas has improved. Identification of specific genomic mutations in key cellular pathways associated with disease progression has led to the development of new potentially highly effective therapies that were specifically targeted against the mutated molecules (Göppner and Leverkus, 2011). The mitogen-activated protein kinase (MAPK) pathway is a signal transduction cascade driven by phosphorylation, which connects growth factors and their respective cell surface receptors in intracellular responses such as cell proliferation (Vidwans et al., 2011). The v-raf murine sarcoma viral oncogene homolog B1 gene (*BRAF*), the most commonly mutated gene in melanoma, encodes a protein kinase in the MAPK pathway. *BRAF* mutations are detected in 30-72% of melanoma cases (Table 1). The most frequent mutation in this gene results in a substitution of thymine for adenine at position 1799 of the gene (T1799A), leading to a change at codon 600 (GTG replaced by GAG), and giving rise to the exchange of valine for glutamic acid at position 600 of the protein (V600E). The V600E mutation represents up to 80% of all mutations described in the *BRAF* gene to date (Davies et al., 2002).

The present study aimed to investigate the frequency of the *BRAF* V600E mutation in cutaneous melanoma patients diagnosed at the Pathology Department of Hospital Araújo Jorge, in Goiânia, Goiás, Brazil. Results were compared to those obtained in different geographical regions worldwide. Possible associations between the presence of the *BRAF* V600E mutation and clinical, pathological, and prognostic parameters in cutaneous melanoma patients (age, gender, distant metastasis, anatomical site, histological type, and Breslow index) were also investigated.

MATERIAL AND METHODS

Selection of melanoma specimens

In the present study, 77 consecutive melanoma patients diagnosed at the Department

of Pathology of the Hospital Araújo Jorge in Goiânia, Goiás, Brazil, during the period of January 2004 to December 2006, were selected. The study protocol was approved by the local Ethics Committee (protocol 049/2010). All melanoma specimens were fixed with formalin and then embedded with paraffin. In order to confirm the diagnosis and to select specimens containing a significant tumor area, selected cases were reviewed by a pathologist. Clinical and pathological information, including age, gender, tumor location, classification of melanoma, Breslow depth, mitotic rate, regional lymph nodes metastasis, and distant metastasis, were obtained from the patients' clinical files. The selected cases were all primary cutaneous melanoma with confirmed pathological diagnoses who presented with clinical and histological parameters. Samples were obtained by surgery, either biopsy or total excision of the tumor, before any radiation therapy or chemotherapy. Based on these criteria, 77 melanoma specimens were included in the study.

Table 1. Literature review of different studies that investigated *BRAF* mutations in melanomas.

Reference	No. of subjects/country	*BRAF* V600E mutation frequency (%)
Davies et al., 2002	34/USA, Italy, Hong Kong, England	55.9
Gorden et al., 2003	77/USA	40.0
Libra et al., 2005	19/Italy	63.0
Goel et al., 2006	58/USA	57.0
Lee et al., 2006	35/USA	60.0
Liu et al., 2007	251/Australia	45.0
Venesio et al., 2008	18/Italy	72.0
Viros et al., 2008	302/USA, Germany, Japan, South Korea	47.0
Casula et al., 2009	35/Italy	31.4
Lázár et al., 2009	74/Hungary	27.0
Narita et al., 2009	71/USA and Australia	39.0
Broekaert et al., 2010	350/Germany, Austria, USA	49.7
Ellison et al., 2010	163/Great Britain	41.1
Scherer et al., 2010	179/Italy and Germany	17.3
Rubinstein et al., 2010	138/USA	69.0
Ellerhorst et al., 2011	223/USA	42.2
Janku et al., 2011	52/USA	50.0
Long et al., 2011	197/Australia	35.5
Shibata et al., 2011	39/Japan	28.2
Menzies et al., 2011	312/Australia	33.6
Lovly et al., 2012	150/USA	30.0

BRAF mutation analysis

Melanoma tissue from every patient was tested for the presence of the *BRAF* V600E mutation using polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP). Sections from archival paraffin-embedded samples were tested at the Laboratory of Genetics and Biodiversity of Pontifícia Universidade Católica de Goías, Goiânia, GO, Brazil. DNA extraction of melanoma samples was accomplished by using the commercial Wizard DNA Isolation Kit (Promega). The *BRAF* V600E mutation was analyzed according to the procedure proposed by Cohen et al. (2003). PCR amplification of exon 15, with *BRAF* exon 5-specific primers (5'-TCATAATGCTTGCTCTGATAGG-3' and 5'-GGCCAAAAATTTAATCAGTGGA-3'), yielded a 224-base pair (bp) product that was then digested with 10 U/µL *Tsp*RI restriction endonuclease (New England Biolabs, Beverly, MA, USA). The *Tsp*RI restriction enzyme digests the 224-bp product resulting in three major bands of 125, 87, and 12 bp from the wild-type allele. The T1799A mutation abolishes a restriction site, and therefore, the digestion results

in a band of 212 bp from the mutant allele and residual bands from the wild type. Independent amplification of exon 5, followed by automated sequencing, validated the results of the *Tsp*RI restriction enzyme assay in five melanoma samples. A melanoma cell line (HTB71) was used as the positive control for the *BRAF* V600E mutation, and normal skin samples were used as negative controls. Automated sequencing was conducted at the Laboratory of Oncogenetics and Radiobiology, Hospital Araújo Jorge, in Goiânia, GO, Brazil.

Statistical analysis

Statistical tests were performed using the Prism GraphPad statistical package (version 5.0). Potential associations between the *BRAF* V600E mutation and patient characteristics were examined by the chi-squared test or the Fisher exact test. A P value less than 0.05 by a two-tailed test was considered to be statistically significant.

RESULTS

A group of 77 patients, including 49 females (63.6%) and 28 males (36.4%) was analyzed. Ten patients (13.0%) were under the age of 45 years and 67 (87.0%) were aged 45 years or older. According to the anatomical site, melanomas were located on the head (15.6%), trunk (36.4%), limbs (18.2%), and feet and hands (29.9%). Tumors located on the trunk, limbs, and feet or hands were considered to be resultant from rare or intermittent ultra-violet (UV) radiation exposure. Melanomas located on the head were considered resulting from chronic exposure to UV radiation. There were 12 (15.6%) patients with chronic exposure and 65 (84.4%) with intermittent sun exposure. According to histological subtypes, melanomas were classified as superficial extensive (42.7%), nodular (26.7%), lentigo maligna (18.7%), and acral or mucosal (12.0%). Two melanomas were designated as unclassifiable. Regarding metastasis, nine (12.0%) patients had metastasis at diagnosis and 66 (88.0%) patients were non-metastatic. Metastasis data were not available for two patients. Clinical, pathological, and prognostic characteristics for the group are described in Table 2.

The *BRAF* V660E mutation was detected in 54 of the 77 cases of cutaneous melanoma, corresponding to a frequency of 70.1%. According to the presence of the *BRAF* V660E mutation, patients were separated into two groups that were compared for their clinical, pathological, and prognostic characteristics, including age, gender, anatomical location, histologic subtype, signs of regression, presence of ulceration, presence of lymphocytic infiltration, presence of microscopic satellites, Breslow depth, presence of metastasis, and sun exposure. No significant statistical association was found between patient characteristics and the presence of *BRAF* V600E mutation (Table 2).

Among patients that were positive for the *BRAF* V600E mutation, 48 (88.9%) were 45 years or older, while six (11.1%) were under 45 years of age. Regarding gender, 37 (68.5%) patients were female and 17 (31.5%) were male. In the mutation group, 8 (14.8%) melanomas were located on the head, 20 (37.0%) on the trunk, 9 (16.7%) were on the limbs, and 17 (31.5%) were on the feet or hands. Considering the anatomical sites of melanomas, we investigated the proportion of mutant cases that could possibly be associated with sun exposure. *BRAF* V600E was positive in 46 (85.2%) cases with intermittent sun exposure sites and in 8 (14.8%) cases with chronic sun exposure. Regarding the presence of metastasis at diagnosis,

47 (87.0%) patients with the *BRAF* V600E mutation were non-metastatic and 7 (13.0%) patients presented metastasis at diagnosis.

Table 2. Univariate analysis of possible associations between *BRAF* V600E mutation and clinical, pathological and prognostic parameters in melanoma patients.

Parameter	V600E (+)	V600E (-)	N[1]	P
Gender				
Male	17	11	28	0.2688
Female	37	12	49	
Age				
<45 years	6	4	10	0.7040
≥45 years	48	19	67	
Histological type				
Superficial spreading	20	12	32	0.3097
Nodular	16	4	20	
Lentigomaligna	9	5	14	
Acral/ mucosal	8	1	9	
Localization				
Head	8	4	12	0.9240
Trunk	20	8	28	
Limbs	9	5	14	
Feet/hands	17	6	23	
Regression signs				
Yes	3	3	6	0.5206
No	48	19	67	
Ulceration				
Yes	22	8	30	0.8766
No	31	14	45	
Lymphocytic infiltration				
Yes	47	22	69	0.3175
No	5	0	5	
Microscopic satellites				
Yes	5	2	7	0.7158
No	47	20	67	
Metastasis				
Yes	7	2	9	0.6810
No	47	19	66	
Sun exposure				
Chronic	8	4	12	0.7750
Intermittent	46	19	65	
Breslow				
<1.0 mm	13	8	21	0.4926
>1.0 mm	41	15	56	

[1]Some cases did not show all the information analyzed by the statical tests. Since they presented most of the information, data were assessed as a whole.

Considering histological parameters, the *BRAF* V600E mutation was detected in 20 (37.7%) cases of superficial spreading melanoma, 16 (30.2%) cases of nodular, nine (17.0%) cases of lentigo maligna, and eight (15.1%) cases of acral or mucosal melanomas. *BRAF* V600E mutations were present in 48 (94.1%) cases of melanomas with signs of regression, in 31 (58.5%) cases without ulceration, 47 (90.4%) melanomas with lymphocytic infiltration, 47 (90.4%) patients who did not have microscopic satellites, and 41 (75.9%) patients with Breslow depths greater than 1.0 mm. However, as shown in Table 2, such differences were not statistically significant.

DISCUSSION

The *BRAF* gene encodes a protein kinase of the MAPK pathway, which is highly

regulated and controls cell division and senescence. When the *BRAF* mutation occurs, the pathway becomes constitutively activated, abolishing Ras activation and ATP requirements. Such deregulation is essential for melanoma cells' proliferation and survival, since the mutant protein is ten times more active than the wild type (Sekulic et al., 2008; Vidwans et al., 2011).

Chemotherapy has only a slight impact on the survival of patients with metastatic melanoma (Hersey et al., 2009; Davar et al., 2011). Until 2011, only two drugs were approved by the US Food and Drug Administration (FDA) for the treatment of metastatic melanoma, decarbazine and interleukin 2, but neither was capable of increasing the overall median survival of melanoma patients (Finn et al., 2012). A phase 3 trial comparing the selective inhibitor of the *BRAF* mutated protein (vemurafenib) with decarbazine in a group of previously untreated melanoma patients demonstrated a significant improvement in the response rate after six months of treatment (84% versus 64%), as well as a reduction in the risk of death (Lemech et al., 2012). These findings represented the basis for vemurafenib FDA approval in the treatment of melanoma in August 2011 (Sullivan and Flaherty, 2011).

As shown in Table 1, the frequency of the *BRAF* V600E mutation varies considerably among different melanoma groups. Therefore, the main objective of our study was to investigate the frequency of the *BRAF* V600E mutation in a group of Brazilian melanoma patients in order to predict whether they might benefit from treatment with the new drug. According to a meta-analysis conducted by Lee et al. (2011), 36 studies investigated *BRAF* mutations in melanoma between 1989 and 2010. *BRAF* mutation frequencies ranged from 22 to 72% in different countries (Lee et al., 2011). Only one study by Jung et al. (2010) was carried out in the southern region of Brazil, revealing a relatively low mutation frequency in this population (39%). Our study detected a frequency of 70%, which is considerably higher, but within the general range identified in the meta-analysis of Lee et al. (2011).

In our study group, superficial spreading melanoma was the most common histological subtype, which is in agreement with previous studies (Ghosh and Chin, 2009). The most common anatomical site was the trunk (36.4%), as described by Dal et al. (2007). One possible explanation for the high number of superficial spreading melanomas that were mainly on the trunk could be intermittent sun exposure as a result of recreational activities, especially those practiced by young people. Melanoma is the second and third most common type of cancer in men and women, respectively, who are between 20 and 29 years of age (Dal et al., 2007; Gosh and Chin, 2009; Siegel et al., 2012).

Our study also aimed to investigate possible associations between the presence of the *BRAF* V600E mutation and prognostic parameters such as Breslow depth, age, and gender, presence of ulceration, lymphocytic infiltration, and distant metastasis. Similar to Qi et al. (2011), we did not find any statistically significant association between the presence of the mutation and histological subtype, gender, or age.

Even though the present data did not demonstrate a statistically significant association between the presence of the *BRAF* V600E mutation and superficial spreading melanoma, as described in other studies (Liu et al., 2007; Lee et al., 2011), there was nonetheless a higher frequency of this mutation in melanomas of this histological subtype (37.7%). These results may be explained by the relatively low frequency of superficial spreading melanomas (32.7%) in our sample.

The association between age and the presence of the *BRAF* V600E mutation in melanomas is controversial. Some studies have shown that this mutation is prevalent in younger

patients (Goel et al., 2006; Liu et al., 2007; Broekaert et al., 2010; Hacher et al., 2010; Menzies et al., 2011; Long et al., 2011). However, this association was not confirmed in the meta-analysis by Lee et al. (2011) or in the present study.

In agreement with Jung et al. (2010), who analyzed 96 Brazilian melanoma patients, no significant association was found between the presence of the *BRAF* V600E mutation and histological parameters, such as the presence of ulceration, signs of regression, and the presence of satellites.

Considering the Breslow depth, the most important parameter for melanoma's prognosis, no significant association between Breslow depths smaller or larger than 1.0 mm and the presence of *BRAF* V600E mutations was observed, thus corroborating the results of Lang and MacKie (2005), Saldanha et al. (2006), and Hacker et al. (2010), as well as the meta-analysis presented by Lee et al. (2011).

In contrast to some previous studies (Saldanha et al., 2006; Liu et al., 2007; Jung et al., 2010), our results did not demonstrate any significant association between the presence of the *BRAF* V600E mutation and chronic or intermittent sun exposure. Hacker et al. (2010) did not detect any difference in the frequency of this mutation between melanomas of the head and neck and those on the trunk and limb; parts of the body that are considered to be exposed to different levels of sun. It is generally accepted that the head and neck receive continuous sun exposure, whereas the trunk and limbs receive only intermittent sun exposure. Qi et al. (2011) also failed to identify an association between this mutation and sun exposure. Therefore, the relationship between sunlight exposure and *BRAF* mutations still requires further research. Studies that demonstrated a significant association between these two parameters have not yet been able to explain the mechanism involved in the mutation, the genes implicated in the event, or the precise contribution of UV radiation in the establishment of *BRAF* V600E mutations (Jou et al., 2012). Mutations recognized as UV radiation signatures are mainly C>T or CC>TT, occurring at dipyrimidine sites, which differs from the *BRAF* V600E mutation (Thomas et al., 2006). Furthermore, *BRAF* V600E mutations are also present in other malignancies, such as papillary thyroid carcinomas, suggesting that factors other than sun exposure could be involved in the mechanism of this mutation.

As described previously (Gorden et al., 2003), our study did not detect a significant association between the *BRAF* V600E mutation and the presence of metastases. One possible explanation could be that this mutation occurs early in melanocyte transformation, contributing to the proliferation and survival of the tumor, but not necessarily contributing to its metastatic spread.

In agreement with results of previous studies (Jung et al., 2010; Long et al., 2011; Lee et al., 2011), we conclude that the *BRAF* V600E mutation should not influence a melanoma patient's prognosis, since no association was found between this mutation and important prognostic parameters, such as Breslow depth, gender, ulceration, age, and histological subtype. However, survival analysis might better define this association. We also concluded that *BRAF* V600E is a very common molecular event in melanoma, and that treatment directed to this mutated protein represents a great promise for melanoma patients, especially for those with metastatic melanoma, who are still waiting for an effective treatment.

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