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The P48T germline mutation and polymorphism in the *CDKN2A* gene of patients with melanoma

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Abstract

CDKN2A has been implicated as a melanoma susceptibility gene in some kindreds with a family history of this disease. Mutations in *CDKN2A* may produce an imbalance between functional p16^{ink4a} and cyclin D causing abnormal cell growth. We searched for germline mutations in this gene in 22 patients with clinical criteria of hereditary cancer (early onset, presence of multiple primary melanoma or 1 or more first- or second-degree relatives affected) by secondary structural content prediction, a mutation scanning method that relies on the propensity for single-strand DNA to take on a three-dimensional structure that is highly sequence dependent, and sequencing the samples with alterations in the electrophoretic mobility. The prevalence of *CDKN2A* mutation in our study was 4.5% (1/22) and there was a correlation between family history and probability of mutation detection. We found the P48T mutation in 1 patient with 2 melanoma-affected relatives. The patient descends from Italian families and this mutation has been reported previously only in Italian families in two independent studies. This leads us to suggest the presence of a mutational “hotspot” within this gene or a founder mutation. We also detected a high prevalence (59.1%) of polymorphisms, mainly alleles 500 C/G (7/31.8%) or 540 C/T (6/27.3%), in the 3' untranslated region of exon 3. This result reinforces the idea that these rare polymorphic alleles have been significantly associated with the risk of developing melanoma.

Key words
- *CDKN2A*
- Familial melanoma
- Mutation
- P48T
- Polymorphism

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The incidence of cutaneous malignant melanoma is increasing all over the world and approximately 10% of melanoma cases are estimated to report a first- or second-degree relative with melanoma (1). Familial melanoma frequently involves multiple primary melanomas, presents clinically atypical moles, and is diagnosed at a younger age than sporadic cases (2). A locus for this hereditary cancer has been mapped on 9p21, and *CDKN2A* (*p16*) is the main candidate gene for melanoma susceptibility. Germline mutations in this gene have been found in some melanoma-prone kindreds (3,4). The *CDKN2A* gene encodes the cyclin-dependent kinase inhibitor p16^{ink4a} involved in cell cycle control. This protein prevents the formation of a functional kinase capable of phosphorylating the retinoblastoma protein and thereby inhibits cell cycle progression from the G1 to the S phase (5).

The likelihood of finding a mutation in

CDKN2A depends on family and population selection, ranging from about 1.5 to 50% (6) and several recurrent mutations in *CDKN2A* (V59G, G101W, 113insR) that have been described are founder mutations (7-9).

Polymorphisms in *CDKN2A* have also been described, but their influence on melanoma risk is uncertain. Debniak et al. (10) found a statistically significant positive association of the A148T variant among patients with malignant melanoma. However, they did not find statistically significant overrepresentation of the 500 C/G and the 540 C/T polymorphisms in the Polish melanoma population. Kumar et al. (11) found that the frequency of the same *CDKN2A* variants at both positions was higher in the melanoma cases, although only the 540 C/T polymorphism was statistically significant. Some intronic mutations also predisposing to melanoma have been described (12).

The etiology of cutaneous melanoma is complex, involving both heterogeneous genetic and environmental components. Three melanoma families have been reported to carry mutations in *CDK4* (3,13). Gillanders et al. (14) studied 49 Australian families and 33 from other continents with at least three melanoma-affected members without *CDKN2A* and *CDK4* involvement. Their research led to evidence for an additional melanoma susceptibility locus on chromosome 1p22.

In order to identify the role of the *CDKN2A* gene in patients with clinical criteria of hereditary melanoma, we conducted a mutant analysis in 22 Brazilian patients with at least one of these criteria: early onset, presence of multiple primary melanoma, or one or more first- or second-degree relatives affected.

This research protocol was submitted to and approved by a National Ethics Committee (Comissão Nacional de Ética em Pesquisa - CONEP) and the patients in the study gave written informed consent to participate. The patients were selected from the Melanoma Outpatient Clinic of the University Hospital, School of Medicine of Ribeirão Preto, with a histopathological diagnosis of cutaneous melanoma and at least one of these clinical criteria: age at diagnosis ≤50 years, presence of multiple primary cutaneous melanomas, or 1 or more first- or second-degree affected relatives. Among the 22 patients selected, 9 were the only melanoma-affected member of the family, 10 had 1 affected relative (first- or second-degree), 2 had 2 affected relatives, and 1 patient had 3 melanoma-affected relatives. Thirteen patients were ≤50 years old and 1 patient had multiple primary melanomas (Table 1). Data concerning the level of UV/sunlight exposure of these patients were not available.

DNA was extracted from peripheral blood using a salting out procedure (15). The three exons were amplified by PCR with the following primers: exon 1, p16F1 (5'-cggagaggg ggagagcaggca-3') and p16R1 (5'-gcgctacct gattccaattc-3'); exon 2, p16F2 (5'-ttcctttccgt catgccgg-3') and p16R2 (5'-gtacaaattctca gatcatcagtcctc-3'); exon 3, p16F3 (5'-gttttctttc tgccctct-3') and p16R3 (5'-cccacatgaatgt gcgctt-3').

Total PCR volume was 25 µL, including 1 µL DNA (0.1 µg/µL), 0.5 µL of each primer (10 pmol), 0.5 µL dNTP (10 mM), 2.0 µL DMSO, 2.5 µL PCR buffer, 0.2 µL Taq polymerase (5 U/µL), 1 µL $MgCl_2$ (50 mM), and 12 µL distilled water. PCR conditions for all exons were as follows: 94ºC for 5 min, 35 cycles of 94ºC for 1 min, 64ºC (exons 2, 3) and 62ºC (exon 1) for 1 min, 72ºC for 1 min, and a final extension step of 10 min at 72ºC.

For secondary structural content prediction (SSCP) analysis 3 µL of PCR products was diluted in 3 µL of denaturing solution (0.015 g bromophenol blue, 0.015 g xylene cyanol, 200 µL 0.5 M EDTA, pH 8.0, and 9.75 mL formamide). The solutions were denatured for 5 min at 94ºC, placed on ice for 2 min and electrophoresed on 8% acrylamide gel at 4ºC. Gels were run at 8 W and 90 V for 8 h and stained with silver nitrate.

The samples with abnormal migration

bands upon SSCP analysis were sequenced using the ABI Prism BigDye® Terminator Sequencing Kit on an automated sequencer 377 (Applied Biosystem, Foster City, CA, USA).

Patients were initially screened for germline mutations of the entire coding sequence of the *CDKN2A* gene by SSCP analysis.

Fourteen patients showed abnormal migration (1 in exon 1, 1 in exon 2, and 3 and 12 in exon 3). DNA sequence analysis of these patients revealed one missense mutation in 1 patient and three polymorphisms in 13 patients (Table 1).

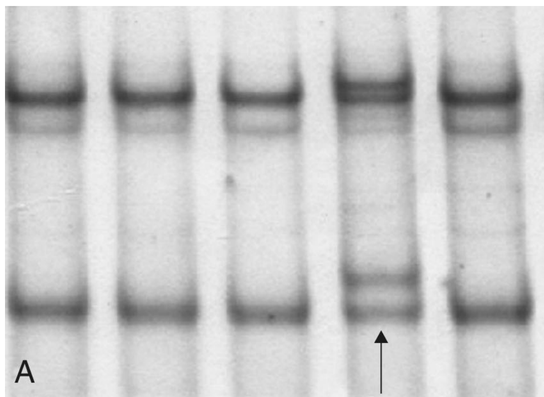
The missense mutation was a C to A transversion in exon 1 at the position 1 of codon 48 in one of the alleles (heterozygosity) which changes the amino acid proline to threonine (P48T) in the $p16^{ink4a}$ protein (Figure 1).

The three polymorphisms were in exons

Table 1. Germline mutations in the *CDKN2A* gene of 22 Brazilian melanoma patients.

Patient No.	Age (years)	Multiple melanomas	Number of relatives affected	Mutation analysis		
				Exon 1	Exon 2	Exon 3
1	57		1	–	–	500 C/G
2	77		1	–	–	–
3	65		1	–	–	–
4	47		–	–	–	500 C/G
5	18		–	–	–	540 C/T
6	53		3	–	–	–
7	58		1	–	–	540 C/T
8	43		1	–	A148T	500 C/G
9	60	2	1	–	–	500 C/G
10	49		–	–	–	540 C/T
11	46		–	–	–	540 C/T
12	50		–	–	–	–
13	45		1	–	–	–
14	36		–	–	–	540 C/T
15	42		–	–	–	540 C/T
16	59		1	–	–	–
17	44		–	–	–	500 C/G
18	52		1	–	–	500 C/G
19	50		2	P48T	–	–
20	43		–	–	–	–
21	52		1	–	–	500 C/G
22	22		2	–	–	–

Mutation analysis: – indicates not detected by PCR.



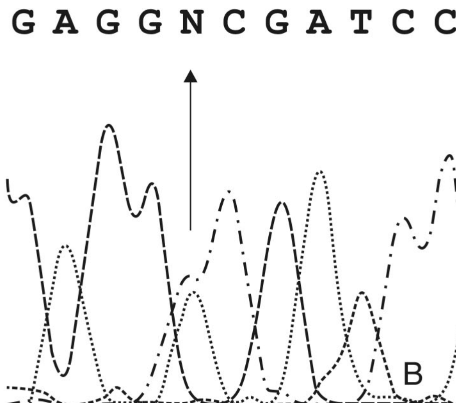


Figure 1. *A*, Secondary structural content prediction analysis of exon 1 shows abnormal migration in patient 19 (arrow). *B*, Sequencing analysis shows heterozygosity (C/A, arrow) at position 142.

2 and 3. The alteration in exon 2 was a G to A substitution at codon 148 (A148T). Thirteen patients (59.1%) had 500 C/G (7/31.8%) or 540 C/T polymorphisms (6/27.3%) in the 3' untranslated region (UTR) of exon 3 (Table 1).

The present study is the first report of germline alterations in the *CDKN2A* gene in Brazilian patients with cutaneous melanoma.

Mutation analysis in 22 patients showed the P48T mutation in 1 patient (No. 19) and three polymorphic alleles in 13 patients mainly in the 3' UTR of exon 3.

The patient carrying the P48T mutation is a man who developed a single primary melanoma at 50 years of age and has 2 first-degree relatives affected. This mutation has been reported before by Moore et al. (16) in an Italian patient with pancreatic carcinoma. Della Torre et al. (17) studied the *CDKN2A* gene in 15 Italian families and found the P48T mutation in one family that segregates with melanoma. In this study (17) the P48T variant of p16 was found to be functionally impaired in its ability to inhibit the cell cycle progression, suggesting a causal role for this mutation. Mantelli et al. (18) found 1 Italian patient with non-familial multiple primary melanoma with this mutation. Our patient descends from Italian relatives and the detection of this same mutation in independent studies suggests that either there are mutational ‘hotspots’ within the *CDKN2A* gene, or the families in these studies are related through ancestry.

The prevalence of *CDKN2A* mutation in our study was 4.5% (1/22). There was a correlation between family history and probability of mutation detection. No mutation was observed when the proband did not have melanoma-affected members (N = 9) or had only one affected relative (N = 10). Similar findings of low *CDKN2A* mutation rates among families with only two affected individuals have been reported by others (4,19). These results support the view that many familial cases represent clusters of sporadic melanomas among high risk phenotypes or less penetrant susceptibility genes (20). Three probands in our study had two or more affected relatives and one of them had the P48T mutation. The prevalence of mutation in these cases would be 33.3% (1/3).

In the present study, we found a high prevalence (59.1% - 13/22) of polymorphisms, mainly alleles 500 C/G (7/31.8%) or 540 C/T (6/27.3%), in the 3' UTR of exon 3. Kumar et al. (11) analyzed these polymorphic alleles in 235 controls and found the 500 C/G allele in 11.7% and the 540 C/T allele in 8.5%. The *t*-test comparing our higher frequency and Kumar et al. (11) controls showed statistical significance ($P < 0.05$) for both polymorphic variants, i.e., the frequencies of these alleles in the patients with melanoma are higher than we would expected at random. In a review study, Hayward (1) reported that these rare polymorphic alleles have been significantly associated with the risk of developing melanoma. According to this investigator, the mechanism by which these variants could confer a melanoma risk is unknown but it is conceivable that these alleles could exert their effect by altering either the stability of the *CDKN2A* transcript or the level of *CDKN2A* transcription; or alternatively, these variants might be in linkage disequilibrium with an as yet unidentified variant directly responsible for increased melanoma susceptibility.

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