New mutation in the CDKN2A gene in brazilian patients with cutaneous melanoma: a pilot study

Nova mutação no gene CDKN2A em pacientes brasileiros com melanoma cutâneo: um estudo piloto

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ABSTRACT
Melanoma is a type of skin cancer very known for its aggressiveness and prognosis detected in the metastatic phases. Several mutations were identified, and in addition to the variations of this disease, predisposition has also been described in the CDKN2A gene. In the present study, 55 individuals had the CDKN2A gene sequenced and evaluated. Only one alteration was highlighted in one individual with melanoma, with this alteration being a substitution of guanine to adenine in position 436 (436 G>A), which affects exon 2 and leads to the alteration in the amino acid aspartate for an asparagine in codon 146 (Asp146Asn) from the CDKN2A gene. This found mutation is different from the ones related to the individuals in Brazil associated with the CDKN2A gene, and it is also not among the most common ones found in different countries and continents.

Keywords: sequencing, DNA, Brazil, skin cancer, melanoma.

RESUMO
O melanoma é um tipo de câncer de pele muito conhecido por sua agressividade e prognóstico detectado nas fases metastáticas. Diversas mutações foram identificadas e, além das variações dessa doença, a predisposição também foi descrita no gene CDKN2A. No presente estudo, 55 indivíduos tiveram o gene CDKN2A sequenciado e avaliado. Apenas uma alteração foi destacada em um indivíduo com melanoma, sendo essa alteração uma substituição de guanina por adenina na posição 436 (436 G>A), que afeta o exon 2 e leva à alteração do aminoácido aspartato por uma asparagina no códon 146 (Asp146Asn) do gene CDKN2A. Essa mutação encontrada é diferente das relacionadas aos indivíduos do Brasil associados ao gene CDKN2A e também não está entre as mais comuns encontradas em diferentes países e continentes.

Palavras-chave: sequenciamento, DNA, Brasil, câncer de pele, melanoma.

1 INTRODUCTION
Skin cancer is one of the most representative types of cancer in Europe, Oceania, North America, and South America [1]. In Brazil, it represents 30% of all malignant tumors and is considered rare in children and black skin adults. It is a pathology that is more prevalent in white skin people, over 40, who are more susceptible to the harmful and cumulative actions of
ultraviolet radiation (UV) and more willing to present skin lesions, which enhance the predisposition to develop the disease [2].

Skin cancer may be separated into two categories: melanoma and nonmelanoma. Nonmelanoma skin cancer (NMSC) is the result of the massive proliferation of keratinocytes and the suppression of the skin inflammatory response [3]. This is the most frequent type of cancer in Brazil and corresponds to approximately 25% of all malignant tumors registered in the country [4]. Skin melanoma (SM) is a type of skin cancer that originates in melanocytes (which are cells that produce melanin, a substance that determines the color of the skin) and presents high lethality. However, it shows a low incidence when compared to NMSC. It has a prevalence in Caucasian adults and represents 4% of malignant skin neoplasms, with this being the most severe due to the high probability of metastasis. Some risk factors contribute to the appearance of SM, such as intermittent or sporadic exposure to the sun; pigmentation characteristics; artificial sun tanning; lack of vitamin D; family history; immune suppression; light skin and presence of nevi, where the risk of melanoma onset is related to the amount of nevi that appear in individuals [5-9].

Of these melanomas, 10 to 15% fit into the category of syndromes of hereditary cancer, being these more prevalent in people from the same family, and in these cases, genetic alterations are passed on through generations. Hereditary melanomas present a high rate of penetrance, and the individual who has the mutation presents a high risk of developing lesions associated with the syndrome throughout life. Some characteristics are associated with this hereditary cancer, such as young age at diagnosis, multiple cases of melanoma in the family, multiple primary melanomas in one individual and other neoplasms (especially pancreatic cancer and tumors of the central nervous system) in the same family, more than one neoplasm in a single individual and multiple generations affected [10-16]. In patients classified under this family category, the risk of developing the disease is from 30 to 70 times higher than in the general population [11,15].

However, melanoma has one of the largest mutational somatic burdens among malignant solid tumors [16]. In 2015, The Cancer Genome Atlas Network (TCGA) published the largest genetic study to date characterizing DNA and RNA alterations and protein analysis of primary or metastatic melanomas and identified 48 main genes related to the appearance of the disease [14, 17].

The CDKN2A (Cyclin-Dependent Kinase Inhibitor 2A) gene, responsible for most hereditary melanomas, acts as a negative regulator of cell proliferation, highly interacting with CDK4 and CDK6. It consists of 3 coding exons, E1, E2, and E3, totaling 30 kb. It encodes
proteins that act in the regulation of the p53 (TP53; 191170) and RB1 (614041) paths, both critical regulatory paths of the cell cycle. Among their coding products, proteins p14ARF and p16INK4A are the most important because they are related to the control of the cell cycle, acting in the mechanisms of tumor suppression [14, 17, 18].

In Brazil, some pathogenic mutations and other still unknown functions were identified by sequencing the CDKN2A gene in families with predisposition to melanoma and not pathogenic variants in individuals without family history [21, 22]. Evidence of germinative mutations related to melanoma with probably European origin has also been found in the population of the Rio Grande do Sul State [20]. Therefore, the present paper analyzes the genetic composition of the CDKN2A gene in a population of the western part of Paraná State with German ascendancy and was founded by immigrants from Rio Grande do Sul State.

2 MATERIAL AND METHODS

2.1 RESEARCH SCENERY

Missal is a city located in southern Brazil and in western Paraná State, colonized during the sixties by Catholic immigrants from German origin, who were previously established in Rio Grande do Sul State, Brazil, which is the Brazilian state with the highest incidence of melanoma when compared to the rest of Brazil. Nevertheless, an important part of the population of Missal presents phenotypic characteristics similar to the European and North American patterns, which are particularly susceptible to melanoma.

According to the last sense, Missal presents a population of 10,474 inhabitants. According to the data of the Network of Health Assistance, available by TABNET (DATASUS), neoplasms occupy second place in the number of ambulatory attendances and hospitalizations in this city, being only after diseases of the respiratory system. Mortality data also point out neoplasm as the second cause of death (25%), with diseases from the circulatory system in the first place (26%). The more prevalent age range regarding the presence and death by neoplasm is from 50-64 years of age (37.5%), followed by 20-49 years of age (19.6%) [25].

2.2 SAMPLE POPULATION AND ETHICAL ASPECTS

This project was approved by the Research Ethics Committee/National Commission for Research Ethics (CONEP)/Brazil Platform, CAAE number 79421517.9.0000.0107.

For the molecular analysis, patients over 18 years of age who were diagnosed with skin cancer (melanoma and non-melanoma) and residents for over three years in the city of Missal were invited voluntarily to participate in the research. Individuals with nonmelanoma skin
cancer and without skin cancer were included as comparison groups. Therefore, for the analysis of population variability of Missal and comparison of patterns, individuals with melanoma (mel group), skin cancer (skin ca group), and healthy people (negative control) were sampled.

Information regarding the socio-demographic profile and life habits was obtained through a questionnaire at the moment of collection of the biological material to verify whether the individual fits into the family melanoma category. The following information was used in the questionnaire: a) individuals with ≥ 3 close family members (parents, siblings, uncles, cousins, grandparents) affected with melanoma; b) individuals with ≥ 3 multiple primary melanomas; c) individuals diagnosed with melanoma at a young age (≤40 years of age); and d) individuals with the presence of pancreatic cancer and melanoma in the family [11, 26-29].

For the DNA sequencing of the regions of interest, 100% of the cases of melanoma in the city were sampled (three men and six women); 38 individuals with skin cancer (21 men and 17 women); and eight individuals without a personal history of cancer (four men and four women), who volunteered to participate in this step of the study, totaling 55 individuals.

For amplification of the CDKN2A gene, the primers CDKN2A, 450 and 930 were used. The primers CDKN2A were used to evaluate whether the population of Missal presents the same genetic information found in the Rio Grande do Sul State [31], and the other primers connect each other in different regions of the CDKN2A gene, with amplicons larger than 400 bp and present variables indicated as pathogenic for melanoma, but not exclusively in Brazil (Table 1).

Table 1. Specifications with regions of interest, sequences of the initiation primers, target exon and size of the expected amplicon after sequencing.

<table>
<thead>
<tr>
<th>Regions of linkage of the initiators</th>
<th>Sequence</th>
<th>Transcribed exon</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDKN2A - Forward 21971092 to 21971092</td>
<td>5'-CTTCCTGGACACGCTGTG-3'</td>
<td>3 P16INK4A</td>
</tr>
<tr>
<td>CDKN2A - Reverse 21970595 to 21970614</td>
<td>5'-AGTCTTCATGCTCCGAGT-3'</td>
<td></td>
</tr>
<tr>
<td>450 - Forward 21995032 to 21995055</td>
<td>5'-TCCGCTCTCTCTCTGAGTGGAA-3'</td>
<td>1 P14ARF</td>
</tr>
<tr>
<td>450 - Reverse 21995528 to 21995507</td>
<td>5'-GGGAGACCGAGAGAACTGA-3'</td>
<td></td>
</tr>
<tr>
<td>930 - Forward 21968490 to 21968509</td>
<td>5'-GAGGGCAGAGAAAGACCGAG-3'</td>
<td></td>
</tr>
<tr>
<td>930 - Reverse 21968993 to 21968969</td>
<td>5'-ACGGGGAGAAAGAAGACTCAATGCAA-3'</td>
<td>3 P16INK4A</td>
</tr>
</tbody>
</table>

Source: Authors

For the molecular analysis, approximately 10 ml of peripheral blood from the volunteers was drawn. Samples were identified and placed in a tube with ethylenediaminetetraacetic acid (ETDA) as an anticoagulant using disposable needles for posterior processing and analysis at the Research Laboratory in Medical Science at UNILA.
For the extraction of DNA, the Invitrogen PureLink Genomic DNA kit was used, following the recommended instructions by the manufacturer. The extracted samples were quantified in a NanoDrop spectrophotometer (Thermo Scientific) following the recommendations of the manufacturer.

The amplification of the regions of interest was performed with a total of 15 μL of a solution having 7.0 μL of MilliQ water, 3 μL of 2 mM dNTP, 1.5 μL of buffer 10X (NH4)2SO4 2 M, Tris-HCl 2 M, MgCl2 1 M and Tween 20 at 1%, 0.6 μL of MgCl (magnesium chloride) 0.6 μL for each initiator at 10 mM/μL, 0.15 μL of Taq DNA polymerase (5 U) and 1.5 μL of DNA (50 ng/μL). The parameters of amplification for the CDKN2A initiators were as follows: 95 °C for 1 minute, 30 cycles of 95 °C for 1 minute, annealing at 60 °C for 1 minute, and 72 °C for 1 minute and 30 seconds. Finally, a final extension of the cycle was performed at 72 °C for 7 minutes. For the initiators 206, 450, and 763, the following PCR patterns were used: 95 °C for 1 minute, 30 cycles of 95 °C for 1 minute, annealing at 57 °C for 1 minute and 72 °C for 1 minute and 30 seconds. Finally, a final extension was performed at 72 °C for 7 minutes.

For initiator 930, the following were used: 95 °C for 1 minute, 30 cycles of 95 °C for 1 minute, annealing at 55 °C for 1 minute, and 72 °C for 1 minute and 30 seconds. Finally, a final extension was performed at 72°C for 7 minutes.

The following reagents were used to perform the sequencing: Thermo Fisher Scientific ExoSAP-IT™ PCR; BigDye™ Terminator v3.1 Cycle Sequencing Kit; precipitation with EtOH/EDTA and the analysis of the sample via ABI-3500 software developed and provided by ThermoFisher.

After the sequencing procedure was performed in the sequencer ABI-3500, the results generated were transferred to a computer for the analysis of the electropherograms and sequences. These analyses were performed using Bioedit and Chromas software.

3 RESULTS

Among all 55 sampled individuals, only one mutation in the CDKN2A gene in one individual belonging to the melanoma group was detected using the primer CDKN2A (Figure 1). This individual presented a substitution of guanine to one adenine in position 436 (436G>A), which affects exon 2 of the CDKN2A gene, switching an Aspartate for one Asparagine in codon 146 (Asp146Asn).
Figure 1. Consensus alignment of the sequences of the nonmelanoma and melanoma (Mel) skin cancer samples using the CDKN2A primer showing a SNP in position 436, which affects exon 2 of the CDKN2A gene, switching an Aspartate for one Asparagine in codon 146 in an individual with melanoma (Mel SNP). The sequencing of the CDKN2A gene is presented on the first line between the groups and was obtained from the NCBI data bank.


4 DISCUSSION

One of the first records of family melanoma was performed by Norris in 1820 in England [30]. Since then, studies have been performed in families with a higher propensity to develop family melanoma [7, 31-35]. Such studies allow evaluation of the degree of susceptibility to melanoma in predisposed individuals and serve as a form of prevention, obtaining a better prognosis and even the early detection of the disease, in addition to developing strategies for better public health management [20, 36, 37]. This information is
highly relevant since family melanoma is a disease with syndrome characteristics, which means that in addition to the predisposition to melanoma, individuals who possess such characteristics may develop other types of tumors, such as pancreatic and tumors of the central nervous system [36].

The main lead gene of family melanoma is CDKN2A, which is present in at least 20% – 40% of families with two or more affected members [41]; however, melanoma is a multifactor disease, and only approximately 10% to 15% of all melanoma cases have hereditary characteristics [21, 22, 32, 34, 39, 40], showing environmental importance in determining most cases.

In the present study, 44% of the melanoma patients fit into the inclusion criteria of family melanoma, having an age of melanoma diagnosis below or equal to 40 years of age. If considering only individuals who possess family members affected by melanoma, the frequency becomes 11% within the normal found mean number [11, 26, 28, 41]. For Australia, Spain and the United Kingdom, the probability of detecting one mutation in people with a family history (≥2 relatives affected) is 25%, with three or more primary melanoma of 29%, or more than one primary melanoma who also has other relatives affected is 27% [42]. In the present study, of the nine melanoma patients analyzed, only one presented a point mutation in the CDKN2A gene (1/9 = 11.11% of the sample), therefore, below the expected mean of 20-40%.

The patient with the point mutation in CDKN2A is male, has German ascendancy, white skin color, does not use tobacco, does not drink alcohol, is an inhabitant of the countryside, performs plantation and creation of animals, does not have or has not defined previous jobs, is exposed to the sun at least five days a week and uses photoprotection at least once a week. The diagnostic age of this patient is 40 years old and presents family members with skin cancer, not specifying whether these relatives have melanoma skin cancer or non-melanoma. In general, the average age at diagnosis of the first melanoma is a factor closely related to the frequency of mutation in CDKN2A in Europe, Australia and the United States of America, with a decrease in frequency with the increase in the average age of diagnosis of melanoma [19, 37].

The highlighted mutation in the present work is an SNP (single nucleotide polymorphism) that refers to the exchange of a guanine base for adenine at position 436 (436G>A), chr9:21970923 (GRCh38.p12). This implies the exchange of the amino acid aspartic acid (ASP) for asparagine (ASN) in codon 146 (p.Asp146Asn). Among the descriptions provided by the NCBI, this mutation is considered a germinate mutation, and it is related to hereditary cutaneous melanoma, however, with uncertain clinical significance [41].
Therefore, since there is not much information on this mutation in the literature, it is interesting that traceability could exist of this mutation in individuals considered relatives of first, second, and even third-degree from this individual to know more about the penetrance of this mutation and its Mendelian characteristics.

The first germinate mutation described with non-coding clinical significance of the CDKN2A gene and the first direct evidence of the non-coding regions in melanoma susceptibility is the c.34 G>T mutation, located in exon 1α. Studies in cells and cultivated tissues have shown functional consequences and progressive diminishing of the expression of the protein p16 [44]. The first evidence found in this mutation was in English patients [42], and in Brazil, evidence of this mutation has also been found in patients with European ascendancy [20, 45]. However, in the present study, this mutation was not evidenced.

Among the other mutations found in Brazil that were also not highlighted in the present study is the p. P48T that leads to an alteration of the protein p16, whose function becomes impossible to execute. This happens because there is an exchange of a cytosine (C) by adenine (A) in the sequence CCG by ACG, leading to a substitution of the proline amino acid by threonine [19]. Additionally, this mutation occurs in exon 1α, affecting only the protein p16 more precisely in the first part of the repetition of ankyrin, a critical location for the inhibition activity of p16 regarding the CDK4 protein [46]. Most of the mutations related to p. P48T were found in families with Italian ascendancy [47, 48]. In Brazil, Hubner and Ramos [19] found this same mutation in a patient with melanoma with Italian ascendancy, indicating a possible hotspot. Nevertheless, in a larger study performed in Latin America related to family melanoma involving the cities of Porto Alegre and São Paulo and in other Latin American countries, this mutation was found in four families, one of them with Italian ascendancy, suggesting a possible founding effect [45].

Another commonly found variant in the population with hereditary melanoma is p. A148T, located in exon 3 and codifies the transcript p16. This mutation leads to the substitution of the guanine base (G) for adenine (A) (GCG /ACG), switching the alanine amino acid for threonine [36]. This mutation is highly controversial regarding its penetrance and its significant clinical value [46, 49, 50]. It is more common in some European countries where patients susceptible to melanoma were found [10, 50, 51], in addition to being the most common in Southern Brazil [36]; however, this was also not evidenced in the present study.

In addition, another frequent mutation that occurs in different countries, such as Italy, the United States, Australia, France, Israel, Spain, and Uruguay, is p. Gly101Trp [10, 52-57]. This mutation affects codon 101 from exon 2, affecting as much the protein p16 as p14 once
both share the same exon. The mutation of the p. Gly101Trp occurs due to a substitution of guanine by thymine (GGG to TGG), which leads to the substitution of glycine to tryptophan in the protein p16 and protein p14. A substitution of the guanine by thymine (CGG to CTG) occurs, switching an arginine for a leucine [55].

Nevertheless, it is highlighted that the most prevalent mutations and the penetrance of the gene CDKN2A in high-risk families vary according to geographic location, being higher in locations with a higher incidence of UV radiation [35, 58, 59]. In Europe, the mutation c.225_243del19 is more prevalent (18 of 89 analyzed individuals = 20.22%), followed by p. R112_L113insR (11 of 89 analyzed individuals = 12.36 %) and p. G101W (10 of 89 analyzed individuals = 11.24%). In Australia, mutations p. M53I (5 from 32 analyzed individuals = 15.63%); p. R24P, p. L32P, c. IVS2-105A>G (totaling 3 from 29 analyzed individuals = 10.34%), and in North America, the mutations c.-34G T (5 from 29 analyzed individuals = 17.24%), p. G101W (4 from 29 analyzed individuals = 13.8%), p. V126D (4 from 29 analyzed individuals = 13.8%) are the most frequent [39]. Nevertheless, in Italy, the mutations p. G23S, p. A36T, p. A60V, p. R80, p. R24P related to CDKN2A was detected in 6/16 (37.5%) of patients with multiple primary melanomas with a family history [60].

The only mutation found in the present study differs from those related to individuals in Brazil associated with the CDKN2A gene [19, 20, 21, 22, 36, 44, 60] and nor is it among the most common mutations found in different countries and continents. This indicates that the pattern of colonization of the region is not the only determinant factor for the onset of melanoma.

5 CONCLUSIONS

Therefore, a single mutation found in the present study differs from those reported to belong to Brazil associated with the CDKN2A gene [19, 20, 21, 22] and is also not among the most common mutations found in different countries and continents, indicating that the colonization pattern in the municipality is not the only determining factor for the appearance of melanoma. Seeing that the majority of melanomas diagnosed at an early stage are curable and that mortality is high among patients diagnosed at more advanced stages, an identification of those genetically predisposed to melanoma is important to direct surveillance actions, early diagnosis and treatment in this group of patients with high risk, expectation of further reduction in morbidity and mortality.
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