Genetic analysis of p16 gene by PCR-SSCP technique and protein p16 expression in oral mucosa and skin melanomas*

*Estudo genético do gene p16 pela técnica de PCR-SSCP e expressão de proteína p16 em melanomas de mucosa oral e melanomas cutâneos**

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Abstract: BACKGROUND - Deletion and mutation of gene CDKN2a, which encodes a specific inhibitor of cyclin-dependent kinase 4 (CDK4), the protein p16, has been regarded as related to cutaneous melanoma tumorigenesis. However, little is known about those alterations in oral mucosa melanomas.

OBJECTIVES – To verify possible p16 gene mutations and its protein expression in sporadic melanomas in oral mucosa and skin.

MATERIAL AND METHODS - 36 primary sporadic melanoma paraffin-embedded specimens (seven oral mucosa and 29 skin lesions) were subjected to molecular analysis of exons 1, 2 and 3 of p16 gene using polymerase chain reaction/single strand conformational polymorphism technique. p16 protein expression was demonstrated by an immunohistochemical technique. Data obtained were correlated with tumor thickness.

RESULTS - Five out of seven oral melanomas, and 17 out of 29 skin lesions displayed signs of alteration in p16 gene molecular analysis. Alterations in exon 2 of p16 gene were the most frequent. Protein p16 expression was observed in only one oral melanoma and in 10/13 (76.9%) skin melanomas up to 1.0 mm-thick and in 7/8 (87.5%) lesions thicker than 1.0 mm.

CONCLUSIONS – Frequency of alterations disclosed by p16 gene molecular analysis in oral mucosa melanomas was 71.42% and 58.6% in cutaneous lesions. The obtained data suggest that p16 gene alterations play a role in the pathogenesis of sporadic melanoma of the oral mucosa. Neither protein p16 expression, nor p16 gene alteration had correlation with tumor thickness.

Keywords: Genes, p16; Immunohistochemistry; Melanoma; Molecular biology

Resumo: *FUNDAMENTOS - A deleção e mutação do gene CDKN2a que codifica um inibidor específico da ciclina dependente de quinase 4, a proteína p16, têm sido implicadas na tumorigênese do melanoma cutâneo. Entretanto, pouco se conhece sobre essas alterações genéticas em melanomas de mucosa oral.*

OBJETIVOS - Verificar a presença de alterações no gene p16 e sua expressão protéica em melanomas esporádicos orais e cutâneos.

MATERIAL E MÉTODOS - Avaliaram-se 36 espécimes de melanoma primário (sete orais e 29 cutâneos). Analisaram-se três exons do gene p16, pela técnica da reação em cadeia da polimerase/polimorfismo conformacional de fita simples do DNA.Verificou-se a expressão tecidual de proteína p16 por técnica imuno-histoquímica. Relacionaram-se os resultados com a espessura dos melanomas cutâneos.

RESULTADOS - Cinco dos sete melanomas orais e 17 dos 29 melanomas cutâneos apresentaram indício de alteração no gene p16. Alterações do exon 2 foram as mais freqüentes, sendo 19 casos nos produtos obtidos com o mesmo iniciador. Observou-se expressão tecidual de p16 em apenas um melanoma oral, em 10/13 (76,9%) casos de melanoma cutâneo de espessura até 1mm e em sete de oito (87,5%) casos de espessura superior a 1mm.

CONCLUSÃO - A freqüência de indícios de alteração na análise genética de p16 nos melanomas de mucosa oral foi de 71,42% e de 58,6% nos cutâneos. É possível sugerir a participação de alterações do gene p16 na patogenia do melanoma esporádico de mucosa oral. Não houve relação da sugestão de alteração genética do gene p16 e de sua expressão tecidual com a espessura dos melanomas cutâneos de diferentes subtipos histológicos.

Palavras-chave: Biologia molecular; Genes p16; Imuno-histoquímica; Melanoma

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INTRODUCTION

Melanoma is the most malignant of cutaneous tumors and it affects predominantly adult individuals, aged between 30 and 60 years. Incidence of cutaneous melanoma, despite improvements in prevention, has increased.[1] In the United States, incidence of this tumor jumped from 1:1,500 individuals in 1953, to 1:100 in 1996.[2]

Approximately 90% of melanomas occur on the skin surface, while almost 2% appear in the mucosae. Most commonly affected sites are head and neck regions. Mucosal melanomas, overall, have an annual incidence of 4 cases per 10 million inhabitants, whereas oral melanomas have an incidence of 1.2 case per 10 million inhabitants/year.[3]

Oral melanoma affects individuals between the sixth and seventh decade of life. The most common anatomic sites are the hard palate, gingiva and alveolar mucosa,[4] representing 0.5% of all oral cavity neoplasias. Recently described series of oral melanomas have small numbers of cases, e.g. the series by Garzino-Demo et al.,[5] with 10 cases in a period of 10 years, and that by Buchner et al.,[6] with five cases, making up a total of 773 solitary oral melanocytic lesions in a period of 19 years.

Deletion and mutation of gene MTS1 (multiple tumor suppressor 1), located at chromosome 9p21, which codes a kinase-dependent cyclin inhibitor 4, p16, have been implicated in the tumorigenesis of melanoma and other neoplasias.[7] Loss of p16 expression has been correlated with progression of familial melanoma.[8] Nevertheless, partial or incomplete loss of p16 expression is also verified in sporadic melanoma.[9]

Little is known about the tumorigenesis of mucosal melanomas of the oral cavity. In an attempt to understand the peculiar mechanisms of this neoplasia, we tried to verify the existence of possible alterations in gene p16, by means of polymerase chain reaction/single strain conformational polymorphism of DNA (PCR-SSCP) technique, and expression of protein p16, by means of Immunohistochemistry, and their correlation to cutaneous melanoma.

SUBJECTS AND METHODS

Thirty-six specimens of primary melanoma were assessed. Seven came from oral mucosa lesions (three from palate, three from palate extending to alveolar border and one from lower lip) and 29 cutaneous lesions (six cases of lentigo maligna melanoma up to 1mm-thick, one case of lentigo maligna melanoma with thickness over 1 mm; five cases of extensive superficial melanoma with thickness up to 1 mm; six cases of extensive superficial melanoma with thickness over 1 mm; four cases of acral lentiginous melanoma with thickness up to 1 mm and seven cases of acral lentiginous melanoma with thickness over 1 mm). None of the patients had family history of melanoma.

p16 genetic analysis

Between 10 and 20 slices measuring 10?m were obtained from paraffin-embedded specimens, collected in glass slides previously washed with absolute alcohol. With the aid of a scalpel blade, tumoral tissue was microdissected from non-stained slices and viewed with a stereoscopic magnifying glass. As a reference, sequential slices were used, stained with hematoxilin-eosin, as a means to aid in the moment of microdissection.[10,11] The material dissected in sterile conditions was then transported to Eppendorf vials for DNA extraction. For this, the slices underwent deparaffinization with xilol under 65ºC and washing in decreasing chain of ethanols.

Material was incubated in lysis buffer (Tris-HCl 1M pH 8.0; EDTA 0.5M pH8; NaCl 1M; SDS 10%) and proteinase K (Invitrogen, Carlsbad, CA, USA) at the concentration of 500μg/μL. Tubes were maintained in bain-marie from three to five days under 55ºC, until complete dissolution if the tissue pellet. Proteinase K was then added (10 to 30μL in 24-hour intervals, and tubes were inverted at least once daily. Enzyme deactivation was performed by exposure to a temperature of 95ºC for a period of 10 minutes.[12,13]

Two hundred μL of ammonium acetate 4M (Synth, BR) were added to the Eppendorf vials, for protein precipitation. Vials were agitated for 20 seconds, in maximal speed, in an ice-filled tub, during five minutes, and centrifuged at 15,000 x g for three minutes. Supernatant containing DNA was transferred for another tube of 1.5 mL. For DNA precipitation, 600μL of isopropanol were added. After homogenization, centrifugation at 15,000 x g for five minutes was performed. Tube pellets were washed with 70% ethanol and centrifuged at 16,000 x g for one minute. After removal of 70% ethanol, DNA pellet was dissolved in 30-50μL of TE buffer (Tris-HCL 10mM, pH 7.4 and EDTA 1mM, pH 8) and maintained under 4ºC until quantification and purity detection in a spectrophotometer for use in PCR.

Once DNA samples from the assessed cases were obtained, spectrophotometric quantification (260/280nm) was carried out in a spectrophotometer (DU 640, Nucleic Acid and Protein Analyzer, Beckman, USA), using 5μL of DNA and 995μL of a TRIS/EDTA solution. Material integrity was viewed in agarose gel at 0.8% (mass/volume) containing 5μL of ethidium bromide (GIBCO BRL, Life Technologies). DNA was then diluted to a final concentration of 100 ng, from

the result obtained regarding DNA concentration, for each case.

The analysis of gene p16 was performed through PCR-SSCP technique.[14]

Primers (Chart 1) were design with a base sequence deposited in the GeneBank (acces number AF527803) using the GeneTool 1.0 software program (BioTools Incorporated, Alberta, Canada), for amplification of a maximum of 200 base pairs (BP) from exons 1, 2 and 3 of p16 gene.

PCR reactions were performed in a thermocycler model PTC-100™ (Programmable Thermal Controller, MJ Research, Inc.). Material was processed for two minutes under 95ºC, followed by 35 or 38 one-minute cycles under 94ºC, one minute between 60ºC and 64ºC (according to primer, Chart 1), and 40 seconds under 72ºC, finishing with an extension of 10 minutes under 78ºC. Amplification of each case was verified with the passing of 10μL of PCR product in 1% agarose gel (1g of NueSieve› GTG› agarose - FMC, BioProducts, Rockland, Manie USA; 1g of Seakem› GTG› agarose - FMC, BioProducts, Rockland, Manie USA; 100μL of TRIS-acetate/EDTA 10X, 4μL of ethidium bromide) contained in an electrophoresis unit (OWL Scientific Plastics, Inc., USA). 5μL of Low DNA Mass Ladder (Life Technologies, Gibco BRL, U.S.) were used to estimate the mass of the case DNA sample and compare band intensity.

Once the amplification of the PCR products was confirmed in agarose gel, polyacrylamide gel electrophoresis was performed (modification of the method described by Maniatis et al.[15]). Gel was used at a concentration of 8% (29.9mL of mili-Q H_2O, 2.6mL of TAE 10X, 5.2mL of 50% glycerol, 14.3mL of 30% acrylamide, 520μL of 10% APS and 52μL of Temed), yielding an effective separation range of 60 to 400bp, thus widening the range of fragment size in the study. PCR product was mixed in a run buffer (98% formamide, EDTA 10mM, 0.05% bromophenol blue and 0.05% xilenocyanol) and put under 95ºC for 10 minutes, for denaturation. After cooling in ice, the mixture was applied into the gel casting gates, set up in the electrophoresis unit. In one of the gates, a molecular weight standard was applied (Low DNA Mass Ladder, Life Tecnologies) for determination of the molecular weight of the amplified fragment. Run was performed with 1X TAE buffer, for approximately five hours, under room temperature (RT). After the end of the run, gel was silver-stained and developed for visualization of DNA bands. Initially, fixation was performed with a solution of 10% ethanol and 0.75% acetic acid during 20 minutes. Following that, staining with 0.2% silver was carried out for 30 minutes, and then washing with mili-Q water for two minutes, developing in a 3% NaOH and 0.3% formaldehyde solution for at least 20 minutes. Reaction was stopped with a solution of 10% acetic acid for 10 minutes , washing with mili-Q water for ten minutes, and drying in cellophane paper. All procedures were done in the absence of light, and with agitation under RT (method modified from Bassan et al.[16]).

In all PCR reactions, were used a positive control (case of oral fibrous inflammatory hyperplasia) and a negative control, constituting of all reactants

CHART 1: Genetic study of gene p16. Sequence of primers used according to exon and number of base pairs

Exon	Primers	Sequence (5´- 3'))	Size (bp)	Cycles	AT (oC)
1	P16G	GGAGGGGCTGGCTGGTCAC CGCACCTCCTCTACCCGACC	181	38	64
	P16F	CGGGGTCGGGTAGAGGAGGTGC GCGCTACCTGATTCCAATCCC	173	35	60
2	P16E	TGGAAACTGGAAGCAAATGTAGG CGCGCCGTGGAGCAGCAG	198	35	60
	P16D	GGAGCCCAACTGCGCCGACCC GCAGCCGCGCGCAGGTACCGT	198	35	64
	P16C	GCGGGGGGCACCAGAGGCAGTAA CCGATTGGCGCGTGAGCTGAGG	186	35	62
3	P16B	CCCCGGTCGCGCTTTCTCTG CGTCCCTACCGGCATTGAAA	195	35	60
	P16A	CAAGAGAGGAGGGCGGGATGTG CTACGAAAGCGGGGTGGGTTGT	197	35	62

bp – base pairs; AT – average temperature

present in the reaction, with no DNA.

Observation and documentation of the reactions was performed with an ultraviolet light transluminator (UV – Fotodyne, INC) and a photographic Polaroid camera (DS 4 – Fischer Biotech), with a Polaroid film type 667. In every gel a bp marker was inserted (Low DNA Mass Ladder, Invitrogen), for standard interpretation.

All gels were scanned and recorded in TIFF and RGB formats, 300 dpis (black-and-white) on AdobePhotoshop 5.0 software. Results for each case were compared to the oral fibrous inflammatory hyperplasia specimen. Analysis was done by three observers, and analysis comparative standards were established (1 to 14). All procedures were carried out in duplicates.

Demonstration of protein p16 expression

Demonstration of a possible expression of protein p16 was done by means of an immunohistochemical technique, with primary monoclonal antibody $p16^{INKa}$ Ab-4, clone 16P04 Labvision (Fremont, CA, USA) diluted to 1/40, preceded by antigenic exposure with citrate buffer pH 6.0, heated by microwaves. Incubation with primary antibody was left overnight, under 4ºC and development of the reaction with the LSAB-DAKO system. Utilized chromogenous was 3,3´-diaminobenzidine with nickel (DAB/Nickel). Countercoloration was omitted in order to make it easier to visualize nuclear reactivity. As a positive control of the reaction, fragments of a nevocellular nevus were used.[17,18] As negative control, primary antibody was replaced by saline buffered with phosphate. p16 expression was considered to be positive when more than 10% of neoplasic cells exhibited nuclear immunostaining upon examation of microscopic fields of x400.[19]

RESULTS

Results of gene p16 molecular analysis from seven oral cavity melanomas and 29 cutaneous tumors are described below and on chart 2. There was no amplification in two specimens of melanoma in the oral cavity.

Exon 1

When migration patterns of primer p16F PCR product bands were analyzed, a single patttern was observed (pattern 10), which was similar to control. With primer p16G, four band migration patterns were observed: pattern 11 (normal pattern similar to control) and patterns 12, 13 and 14 (patterns suggesting alterations). Oral mucosa melanomas displayed pattern 13, and cutaneous tumors, regardless of their thickness, displayed patterns 12, 13 and 14 of band migration (Figure 1).

Exon 2

Three band migration patterns were verified with primer p16C: pattern 5 (normal pattern similar to conrol) and patterns 6 and 7 (patterns suggesting alterations). Mucosal and extensive superficial melanomas exhibited migration patterns 6 and 7 lentigo maligna melanoma and acral lentiginous subtypes exhibited solely migration pattern 6. Patterns 8 and 9, regarding primers p16D and p16E, respectively, were similar to patterns displayed by control (Figure 2).

Exon 3

With primer p16A, the same band migration pattern (similar to control – pattern 1) was ound for both oral mucosa and cutaneous tumors, thus with no signs of alteration in this region of the gene. With primer p16B, three band migration patterns were observed: pattern 2 (similar to conrol), and patterns 3 and 4 (patterns suggestive of alteration). Pattern 3 was observed in cases of lentigo maligna melanoma with thickness below or equal to 1mm. Pattern 4 was found in melanomas of the extensive superficial type, regardless of thickness. All mucosal tumors exhibited band migration pattern 2 (Figure 3).

Demonstration of protein p16 expression

For positive control represented by nevocellular nevus, p16 expression had a strong nuclear staining pattern, and, ocasionally, cytoplasmatic staining (Figure 4A). Cytoplasmatic immunostaining was not considered as being positive p16 expression in the studied cases.

Of the five mucosal melanomas, only one (case 30) displayed nuclear immunostaining in approximately 30% of neoplasic cells (Figue 4B).

Of the 21 cases of cutaneous melanoma submitted to immunohistochemistry for demonstration of protein p16, 17 presented nuclear immunostaining that was considered as positive (i.e. over 10% of stained neoplasic cell nuclei). Of the six cases of up to 1mm-thick lentigo maligna melanoma, five presented p16 expression. The only case of lentigo maligna melanoma which had over 1mm of thickness did not stain positevely. Four out of five cases of thin (thickness of up to 1mm) extensive superficial melanoma expressed protein p16. The four cases of over 1mm-thick extensive superficial melanoma were marked for p16. One out of two cases of acral lentiginous melanoma with up to 1mm of thickness exhibited immunostaining for p16 (Figure 4C), and all three cases of thick (thickness over 1mm) acral lentiginous melanoma expressed protein p16 (Figure 4D).

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CHART 2: Molecular analysis of p16 by means of PCR-SSCP technique and demonstration of p16 expression by means of immunohistochemical techniques in both oral and cutaneous melanomas

		EXON 3		EXON 2			EXON 1		Proteon p16
Type	**Cases**	**p16A**	**p16B**	**p16C**	**p16D**	**p16E**	**p16F**	**p16G**	
Oral	7	NA	P2	P6*	P8	NA	NA	NA	Neg
Melanoma	8	P1	P2	P7*	P8	P9	P10	P13*	Neg
	9	P1	NA	P6*	NA	P9	NA	NA	NP
	18	NA	NA	NA	NA	NA	NA	NA	NP
	29	P1	P2	P6*	P8	P9	P10	NA	Neg
	30	P1	P2	P6*	P8	P9	P10	NA	Pos
	36	NA	NA	NA	NA	NA	NA	NA	Neg
L1	5	P1	P2	P6*	NA	P9	P10	P12*	Pos
	6	P1	P3*	NA	NA	NA	NA	P13*	Pos
	10	NA	NA	P5	NA	NA	NA	NA	Neg
	11	P1	P2	P6*	NA	NA	NA	NA	Pos
	15	NA	NA	NA	NA	NA	NA	P11	Pos
	21	P1	P2	P6*	P8	P9	NA	P14*	Pos
L2	4	P1	P2	NA	P8	P9	P10	NA	Neg
S1	14	P1	P4*	P6*	NA	P9	P10	P13*	Neg
	17	P1	P2	P5	P8	P9	P10	P11	Pos
	19	P1	P4*	P7*	P8	NA	P10	P12*	Pos
	22	P1	NA	P5	P8	P9	P10	P14*	Pos
	23	P1	NA	P5	P8	P9	P10	P11	Pos
S2	3	P1	P2	P5	P8	P9	P10	P11	Pos
	13	P1	P4*	P6*	P8	P9	P10	P13*	Pos
	20	P1	P4*	P7*	NA	NA	P10	P12*	Pos
	24	P1	NA	P5	P8	P9	P10	P11	Pos
	27	P1	P2	P5	NA	P9	P10	NA	NP
	28	P1	NA	P6*	NA	NA	P10	NA	NP
A1	16	P1	NA	P6*	NA	NA	NA	P12*	Neg
	32	P1	P2	P6*	NA	P9	P10	NA	Pos
	33	P1	P2	P5	NA	P9	P10	NA	NP
	35	NA	NA	NA	NA	NA	NA	NA	NP
A2	1	P1	P2	P6*	P8	P9	P10	NA	NP
	2	NA	NA	P5	P8	NA	P10	NA	Pos
	12	NA	NA	P6*	NA	NA	NA	NA	NP
	25	P1	NA	NA	NA	NA	P10	P12*	Pos
	26	P1	P2	P6*	NA	P9	P10	NA	Pos
	31	P1	P2	P6*	NA	P9	P10	NA	NP
	34	NA	P2	NA	NA	P9	NA	NA	NP

L1: Lentigo maligna melanoma thinner than or equal to 1mm; L2: Lentigo maligna melanoma thicker than 1mm; S1: Extensive superficial melanoma thinner than or equal to 1mm; S2: Extensive superficial melanoma thicker than 1mm; A1: Acral lentiginous melanoma thinner than or equal to 1mm; A2: Acral lentiginous melanoma thicker than 1mm; NA: not amplified; P1: Pattern 1 (similar to control); P 2: Pattern 2 (similar to control); P 3* (sign of alteration); P 4* (sign of alteration); P 5: Pattern 5 (similar to control); P 6*: Pattern 6 (sign of alteration); P 7*: Pattern 7 (sign of alteration); P 8: Pattern 8 (similar to control); P9: Pattern 9 (similar to control); P10: Pattern 10 (similar to control); P11: Pattern 11 (similar to control); P12*: Pattern 12 (sign of alteration); P13*: Pattern 13 (sign of alteration); P14*: Pattern 14 (sign of alteration). NP: not performed. Control of p16 molecular analysis: DNA extract from a specimen of fibrous inflammatory hyperplasia of the oral mucosa. Pos: presence of p16 expression; Neg: absence of p16 expression.

p16 expression, or its absence (by means of immunohistochemistry), are compared to signs of alteration observed on molecular analysis (Chart 1), we observe that: three of the four cases of oral mucosa melanomas with absence of protein p16 expression, upon histochemical assessment, displayed alterations on the molecular analysis of gene p16. The case with p16 tissue expression had signs of alteration in the analysis of exon 2 (p16C). Of the 17 specimens of cutaneous melanoma with p16 expression, as evidenced by the immunohistochemical technique, 11 had signs

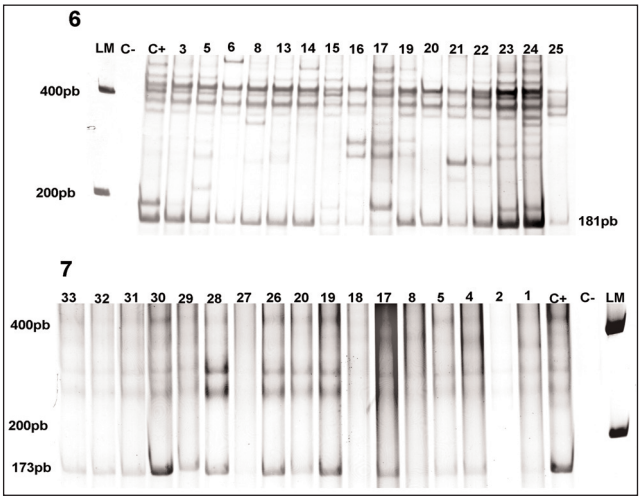

FIGURE 1: Molecular analysis of gene p16 by means of the PCR/SSCP technique, with primers of exon 1: 6) p16G – pattern 11 (similar to control): cases 3, 15, 17, 23, 24; pattern 12: cases 5, 16, 19, 20, 25; pattern 13 (suggestive of alteration): cases 6, 8, 13, 14; pattern 14 (suggestive of alteration): cases 21, 22. 7) p16F – all cases with pattern 10 (similar to control). LM: Low Mass; Bp: Base pairs; C: control

of p16 alterations on the molecular analysis. Of the four cases of cutaneous melanoma with absence of p16 expression, two presented signs of alteration upon molecular analysis (cases 14 and 16).

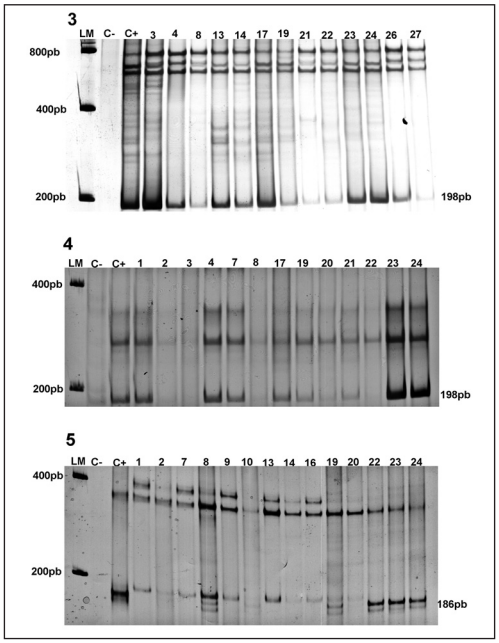

FIGURE 2: Molecular analysis of gene p16 by means of the PCR/SSCP technique, with primers of exon 2: 3) p16E – all with pattern 9 (similar to control); 4) p16D – all with pattern 8 (similar to control); 5) p16C – pattern 5 (similar to control): cases 2, 22, 23 and 24; pattern 6 (suggestive of alteration): cases 1, 7, 9, 13, 14, 16 and 20; pattern 7 (suggestive of alteration): cases 8 and 19. LM: Low Mass; Bp: Base pairs; C: control (inflammatory fibrous hyoerplasia); C-: negative control

DISCUSSION

Molecular analysis of p16 revealed that part of the analyzed tumors (71.42% of mucosal melanomas and 58.6% of cutaneous melanomas) presented signs of alterations on exons 1, 2 and 3 (see chart 1). Exon 2 was the one that most often presented signs of alteration, both for cutaneous and mucosal tumors.

Five out of seven (71.42%) studied mucosal melanomas revealed a pattern suggestive of alteration on exon 2 (p16C), four of the cases displaying pattern 6, and one, 7. One case also presented signs of alteration on exon 1 (p16G – pattern 13).

Mucosal melanomas are very rare and represent only 0.5% of oral cavity neoplasias. Recently described series of oral melanomas present only a small number of cases.[5,6] Regarding p16 genetic studies in this type of melanoma, there is just the work by Chang et al.,[20] who carried out a genetic study of p16 in a melanoma strain obtained from a single case of primary melanoma of the palate. They observed an absence of p16 transcription, by means of analysis with RT-PCR technique, in the cell lineage obtained from the primary tumor. Results obtained in the present study show that the frequency of signs of alteration in the genetic analysis of p16, with the presently employed methodology, is high (71.42%). This piece of data suggests a participation of possible gene p16 alterations in the pathogenesis of sporadic melanoma of the oral mucosa.

One of the seven specimens of oral melanoma (case 36) did not have amplification of analyzed

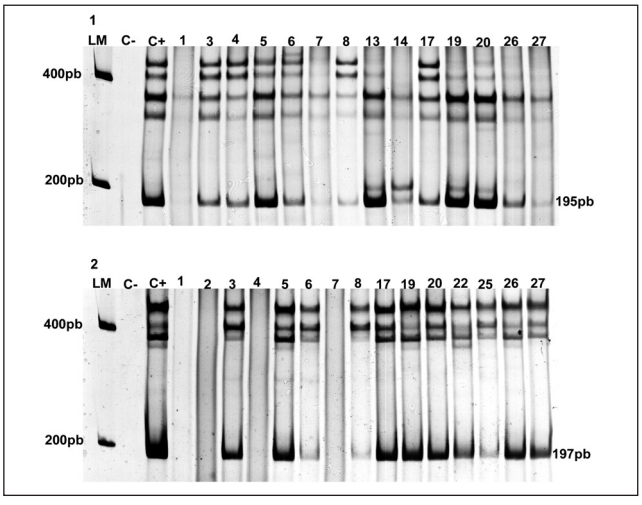

FIGURE 3: Molecular analysis of gene p16 by means of the PCR/SSCP technique, with primers of exon 3: 1) p16B – pattern 2 (similar to control): cases 3, 4, 5, 7, 8, 17, 26, 27; pattern 3 (suggestive of alteration): case 6; pattern 4 (suggestive of alteration): cases 13, 14, 19, 20. 2) p16A – pattern 1 (similar to control): cases 3, 5, 6, 8, 17, 19, 20, 22, 25, 26, 27. LM: Low Mass; Bp: Base pairs; C: control (inflammatory fibrous hyoerplasia); C-: negative control

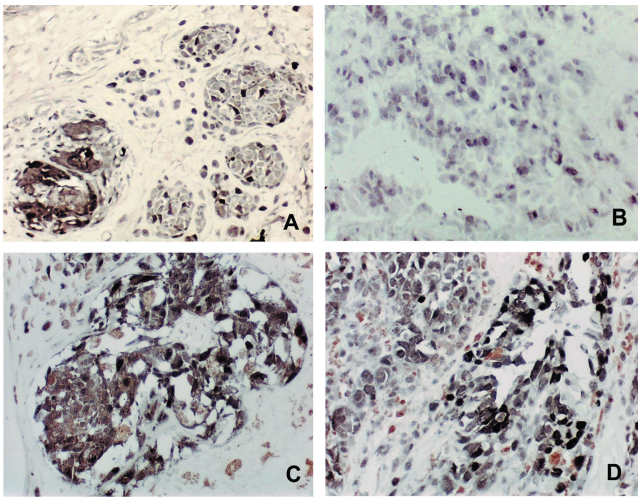

Figure 4: Demonstration of protein p16 expression by immunohistochemical technique with anti-p16 monoclonal antibody. **A –** Nevocellular nevus (positive control). Immmunostained nuclei in black and slight cytoplasm staining. **B –** Oral melanoma (case 30). Nuclear immunostaining of neoplasic melanocytes. **C –** Thin cutaneous melanoma (case 32). Atypic melanocyte nuclei immunostained in black. **D –** Cutaneous melanoma with thickness over 1mm (case 12). Nuclear immunostaining in black and brown cytoplasmatic melanic pigment

sequence. This may be due to the fact that we utilized material recoverd from specimens submitted to routine histological technique (fixed in formaldehyde and embedded in paraffin), thus the possibility of interference during sample processing.

Of the 29 cutaneous melanoma, 14 cases were observed to have signs of exon 2 alteration (p16C), 12 of those cases with pattern 6, and two with pattern 7. Five out of 29 cases of cutaneous melanoma displayed signs of alteration on exon 3 (p16B), one with pattern 3 and four with pattern 4. Signs of exon 1 alteration were observed in 10/29 cases of cutaneous melanomas (five cases with oattern 12, three with pattern 13, and two with pattern 14).

When signs of alteration are correlated to histological type and microstaging of cutaneous melanomas, four cases of lentigo maligna melanoma of up to 1mm of thickness are verified to have signs of alteration (two cases for exons 2 and 1; one case for exons 3 and 1; and one case for exon 2). The single case of over 1mm-thick malignant lentigo melanoma did not exhibit any sign of alteration upon p16 genetic analysis. Two cases of lentigo maligna melanoma of the extensive superficial type with up to 1mm of thickness presented signs of alteration with exons 3,2 and 1, as well as two cases of the same histological type with over 1mm of thickness. In this last group, one case displayed sign of p16 exon 2 alteration. Acral lentiginous melanoma group had signs of alteration for exons 1 and 2; being that one case with up to 1mm of thickness had alterations on both exon 1 and 2, and one only on exon 1. Five cases of acral lentiginous melanoma which were over 1mm-thick presented the following pattern of alteration: four on exon 2, and one on exon 1.

Briefly, p16 genetic analysis of cutaneous melanomas revealed signs of alteration in four out of six (66%) cases of lentigo maligna melanoma up to 1mm-thick; three out of five (60%) cases of malignant melanoma of the extensive superficial type of up to 1mm of thickness; three out of six (50%) cases of over 1mm-thick extensive superficial melanoma; two out of four (50%) acral lentiginous melanoma of up to 1mm of thickness and five out of seven (71.4%) acral lentiginous melanoma measuring over 1mm in thickness. Overall frequency of alterations in the studied cases of cutaneous melanoma was 58.6% (17 out of 29 cases).

Considering all studied cases of melanoma (n=36), a higher frequency of p16 exon 2 alterations was verified (19 out of 36 cases, frequency of 52.77%), followed by exon 1 (11 out of 36 cases, frequency of 30.55%) and exon 3 (five out of 36 cases, frequency of 13.88%). Lamperska et al.[21] reported that the majority of p16 mutations described in malignant melanoma are in exon 2.

The present study is composed by cases of sporadic melanoma, i.e., with no history of familial malignant melanoma.Gene p16 is considered to be the gene of suceptibility to melanoma, due to its frequent inactivation in familial malignant melanomas.[7] However, partial or incomplete loss of p16 expression is also verified in sporadic melanomas.[9] Other authors, nevertheless, report that gene CDKN2A mutation is little frequent in malignant sporadic melanoma.[22-24]

In this study, no correlation was observed between the frequency of signs of p16 alteration with cutaneous lesion thickness. Melanomas measuring up to 1mm in thickness from different histological types presented frequency of signs of alteration of 60% (nine out of 15 cases), and in those measuring over 1mm, such frequency was of 50% (seven out of 14 case). Cachia et al.[11] did not observe any anomalies in p16 analysis by means of PCR-SSCP when comparing two groups of primary malignant melanoma (39 cases), with thickness of up to 0.75mm (19 cases) and over 3mm (20 cases). They concluded that p16 gene mutation is rare both in thin and thick melanomas. These authors performed tissue microdissection with a technique similar to the one used in the present study. They highlighted that the possibility of material contamination by normal material in the surrounding tissues is below 25% for thin tumors, and below 10% for thick ones.

We chose to compare melanomas of up to 1mm in thickness with those with over 1mm, once this microstaging measure is considered as the cutoff line of therapy, in what regards safety margins and search for sentinel lymph node.[25]

Observed p16 expression, by means of immunhistochemistry, showed a pattern in nuclear and cytoplasmatic staining in positive control (nevocellular nevus). This pattern of expression is related to the wild form of protein p16.[18] Nevertheless, cytoplasmatic positivity was not considered for semiquantitative analysis of the studied cases. p16 expression was observed only in one case of oral melanoma (case 30). On the other hand, Tanaka et al.[19] verified tissue p16 expression in seven out of 13 cases of oral melanoma.

In cutaneous melanomas, no differences were found in p16 protein expression among thin cases (10 out of 13, or 76.9 %) and thick melanomas (seven out of eight, or 87.5%). Such results are in agreement with those of Ghiorzo et al.,[18] which also did not find difference in p16 expression as *in situ* melanomas were compared, both primary invasive and metastatic ones.

In some cases of cutaneous melanoma, some cases of cytoplasm immunostaining by p16, besides the nuclear one, were observed, similarly to what was found by Radhi[26] and Mihic-Probst et al.[17] In normal cells there is both nuclear and cytoplasm p16 expression. Nuclear expression would be related to probable alele mutation of the p16 gene.[18]

As expression or lack of expression of p16 results are compared with signs of change observed by means of molecular analysis in the same tumors, p16 protein expression is observed concomitantly with evidences of change in the molecular analysis of the p16 gene in 12 cases (one mucous and 11 cutaneous). On the other hand, absence of p16 protein expression was observed in six cases that presented signs of alteration of p16 gene by molecular analysis (four mucosal and two cutaneous). Six cases with protein p16 expression did not exhibit signs of gene p16 alteration on molecular analysis.

Literature refers that loss of gene p16 expression is frequent in cutaneous malignant neoplasias.[27] Lack of p16 expression could constitute evidence of gene p16 mutation or deletion.[28] Loss of p16 expression is more often observed in thicker melanomas and in metastases.[29,30] In this study, we verified that in thicker tumors p16 expression was noted in seven of the eight cases, four of them being simultaneous to signs of alteration in gene p16 on molecular analysis..

CONCLUSION

Frequency of signs of alteration in p16 genetic analysis in oral mucosa melanomas was 71.4%. In studied cutaneous melanomas, frequency was 58.6%. It may be suggested that of gene p16 alterations play a role in the pathogenesis of sporadic oral mucosa melanoma. There was no relation between signs of alteration of p16 and the level of invasion by cutaneous melanomas of different histological subtypes. Tissue expression of p16 also did not correlate to lesion thickness. It was noted in 10 out of 13 (76.9%) cases of cutaneous melanoma up to 1mm in thickness

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