BRUNO AUGUSTO BENEVENUTO DE ANDRADE

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UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

Bruno Augusto Benevenuto de Andrade

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Orientador: Prof. Dr. Oslei Paes de Almeida

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Este exemplar corresponde à versão final da tese defendida pelo aluno Bruno Augusto Benevenuto de Andrade, e orientada pelo Prof. Dr. Oslei Paes de Almeida.

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RESUMO

O melanoma bucal é um tumor potencialmente agressivo de origem melanocítica, que surge através de uma lesão melanocítica benigna ou de melanócitos da mucosa. Em melanomas é conhecido que a transformação de melanócitos em células de melanoma decorre de alterações nos mecanismos de controle do ciclo celular. Nesse caso, as alterações mais comuns são a superexpressão de ciclina D1e Skp2 e mutações ou deleções de p16, p21 e p27. A avaliação do ciclo celular usando anticorpos contra proteínas nucleares envolvidas na regulação da replicação de DNA também ganhou interesse especial na tentativa de predizer o comportamento biológico em tumores malignos e diferenciação entre lesões benignas e malignas. O objetivo desse trabalho foi avaliar a expressão imunoistoquímica de ácido graxo sintase (FASN) e de proteínas envolvidas nos mecanismos de controle e progressão do ciclo celular como p16, p21, p27, ciclina D1 e Skp2, além dos marcadores de proliferação celular Mcm-2, Ki-67 e geminina em nevos intramucosos e melanomas primários de boca. Os resultados mostraram que FASN, p21, ciclina D1, Skp2, Ki-67, Mcm-2 e geminina foram negativos ou raramente expressos nos casos de nevo, enquanto que nos casos de melanoma, observou-se alta expressão dessas proteínas. Esses marcadores podem estar envolvidos na patogênese do melanoma bucal, podendo eventualmente ser utilizados como ferramenta diagnóstica adicional para ajudar no diagnóstico diferencial entre lesões melanocíticas benignas e malignas.

Palavras-chave: melanoma, nevo, diagnóstico, ácido graxo sintase, ciclo celular, proliferação celular, imunoistoquímica.

ABSTRACT

Oral melanoma is a potentially aggressive tumor of melanocytic origin, which arises from a benign melanocytic lesion or mucosal melanocytes. In melanomas it is known that the transformation of melanocytes in melanoma cells is caused by alterations in the mechanisms of cell cycle control. In this case, the most common changes are the overexpression of cyclin D1 and Skp2 and mutations or deletions of p16, p21 and p27. The evaluation of the cell cycle using antibodies against nuclear proteins involved in the regulation of DNA replication has also gained particular interest in the effort to predict the biologic behavior and to differentiate between benign and malignant lesions. The aim of this study was to evaluate the immunohistochemical expression of fatty acid synthase (FASN) and proteins involved in the mechanisms of control and cell cycle progression such as p16, p21, p27, cyclin D1 and Spk2, and the cell proliferation markers Mcm-2, Ki-67 and geminin in intramucosal nevi and oral primary melanomas. The results showed that FASN, p21, cyclin D1, Skp2, Ki-67, MCM-2 and geminin were negative or rarely expressed in the cases of nevi, whereas in the cases of melanoma, it was observed a high expression of these proteins. These markers may be involved in the pathogenesis of oral melanoma and may eventually be used as additional diagnostic tool to help in the differential diagnosis between benign and malignant melanocytic lesions.

Key words: melanoma, nevi, diagnosis, fatty acid synthase, cell cycle, cell proliferation, immunohistochemistry

SUMÁRIO

INTRODUÇÃO

Melanoma bucal

O melanoma é um tumor potencialmente agressivo de origem melanocítica. Somente 1% de todos os melanomas se desenvolve em mucosa bucal e estes correspondem a 0,5% de todas as malignidades bucais e menos de 0,01% de todas as biópsias bucais (Gu *et al*., 2003; Hicks & Flaitz, 2000). As regiões mais freqüentemente afetadas pelo melanoma bucal são o palato e a gengiva maxilar, ambos correspondendo a 80% dos casos. Outros sítios de acometimento incluem gengiva mandibular, mucosa bucal, soalho de boca e língua (Rapini *et al*., 1997; de Andrade *et al.,* 2012).

A idade dos pacientes afetados varia de 20 a 80 anos, com média de 55 anos, e alguns autores demonstram predominância maior em indivíduos do sexo masculino (Hicks & Flaitz, 2000; de Andrade *et al.,* 2012). Os sinais e sintomas iniciais são normalmente aumento de volume assimétrico, de contorno irregular que é geralmente pigmentado (Hicks & Flaitz, 2000). Essa pigmentação pode se apresentar uniforme com coloração marrom ou negra, ou demonstrar variação de cores como preto, marrom, cinza, roxo e vermelho, além de áreas sem pigmentação, o que é normalmente raro (Gu *et al*., 2003; Umeda *et al*., 2008). Histologicamente é composto de ninhos ou ilhas de melanócitos, que são arranjados em padrão organóide, alveolar ou sólido. As células malignas do melanoma podem demonstrar ampla variação de forma, incluindo aspectos fusiformes, plasmocitóides, de células claras e epitelióides (Barker *et al*., 1997; de Andrade *et al.,* 2012).

A etiologia do melanoma bucal não é bem conhecida. Considera-se que o melanoma bucal tenha fatores etiológicos semelhantes ao melanoma cutâneo, incluindo exposição solar, embora no palato e gengiva seja difícil de ser considerado e presença de displasia névica (Hicks & Flaitz, 2000; Gu *et al*., 2003). Muitos melanomas bucais surgem *de novo*, a partir de uma mucosa aparentemente normal, mas aproximadamente 30% são precedidos por

pigmentações bucais de muitos meses ou até anos (Rapini *et al*., 1985; Sortino-Rachou *et al*., 2009). Algumas dessas lesões precursoras são constituídas por melanócitos citologicamente atípicos que podem constituir de fato, uma fase macular pré-invasiva do melanoma (Hicks & Flaitz, 2000).

História familiar, síndromes, fatores de crescimento e proliferação (como bcl-2 e Ki-67), defeitos citogenéticos e mutações nos genes supressores de tumor p16 e p19 também influenciam na formação do tumor (Hicks & Flaitz, 2000; Gu *et al*., 2003). A importância das mutações nos genes supressores de tumor e da desregulação dos fatores de crescimento para o surgimento do melanoma são ilustrados por estudos de cultura celular comparando melanócitos, células névicas e células tumorais do melanoma (Healy *et al*., 1995; Kamb *et al.,* 1997). A maioria das células tumorais do melanoma possui habilidade de invadir ágar gel, enquanto que menos de 0,1% dos melanócitos e células névicas invadem. Esses achados ao correlacionar as células tumorais do melanoma, os melanócitos e as células névicas podem ser atribuídos aos efeitos das mutações dos genes supressores de tumor ou por proliferação de fatores de crescimento (Healy *et al*., 1995; Kamb *et al.,* 1997).

Estudo realizado por Woenckhaus *et al.* (2004) demonstrou que a perda de heterogozidade no cromossomo 12p13 e perda de expressão da proteína p27 contribui para a progressão do melanoma de pele. A maior parte (90%) dos casos de melanoma é esporádica, mas o conhecimento da base genética dos casos familiais de melanoma tem ajudado na compreensão da patogênese molecular desta doença (Ibrahim & Haluska 2009). A base molecular do melanoma começou a ser esclarecida em meados de 1990, quando foram observadas mutações no gene regulador do ciclo celular *CDKN2A* (cyclin-dependent kinase inhibitor 2A), localizado no cromossomo 9p21(Lin *et al.,* 2008). *CDKN2A* codifica duas proteínas inibidoras de quinase, p16 e p14 (Ibrahim & Haluska 2009). Conforme descrito anteriormente, p16 inibe cdk4/6, responsáveis pela fosforilação da proteína retinoblastoma (Rb) que, no seu estado hipofosforilado ativo, liga-se a E2F e inibe

a progressão do ciclo celular (Lin *et al.,* 2008). Já p14 promove a estabilização da proteína supressora de tumor p53, prevenindo sua degradação mediada por Mdm2 (Lin *et al.,* 2008).

Mutações em cdk4 também têm sido relacionadas ao desenvolvimento do melanoma de pele, conferindo resistência à atividade inibidora de p16 (Lin *et al.,* 2008). Além disso, sabe-se que a transformação de melanócitos em células de melanoma decorre de alterações nos mecanismos de controle do ciclo celular, mais especificamente em p53 e Rb (Li *et al.,* 2006). Nesse caso, as alterações mais comuns nos mecanismos que envolvem a ação de Rb são a superexpressão de ciclina D1, superexpressão e mutações em cdk4 ou mutações e redução na expressão de p16 (Li *et al.,* 2006; Fecher *et al.,* 2009).

Nevo melanocítico bucal

Nevos melanocíticos bucais (NMBs) são tumores benignos de células névicas, células que possuem origem na crista neural, encontradas na pele e em mucosas de revestimento, incluindo a mucosa bucal (Meleti *et al*., 2007). Enquanto existe um grande número de casos relatados de nevos melanocíticos cutâneos, os NMBs são raros. Na literatura existem aproximadamente 120 artigos sobre NMBs publicados entre os anos de 1965 e 2005, sendo a maioria destes, relatos isolados de casos clínicos ou estudos de pequenas séries de casos (Mackie *et al.,* 1985; Buchner *et al*., 2004). Em uma revisão da literatura realizada por Buchner *et al.* em 2004, eles identificaram menos de 300 casos de NMBs relatados, sendo que destes, foram adicionados mais 91 casos de seu estudo.

A prevalência e incidência do NMB não têm sido sistematicamente estudada. No estudo de Buchner *et al.* (2004), os NMBs representaram cerca de 0.1% das 90.000 biópsias analisadas durante um período de 19 anos. A relação mulher/homem é de aproximadamente 1.5:1. Não existe predileção por raça e a idade no momento do diagnóstico varia de 3 a 85 anos (Buchner *et al.,* 2004; Meleti *et al.,* 2007).

Clinicamente, os NMBs são tipicamente vistos como máculas redondas ou ovais bem circunscritas, com tamanho variando de 0.1 a 3 cm, podendo ocorrer isoladamente ou como múltiplas lesões. Sua cor pode variar do marrom ao azul, azul escuro ou negro, sendo rara a presença de nevos não pigmentados (Grichnik *et al.,* 2005; Meleti *et al.,* 2007). O palato duro, mucosa jugal e gengiva são os locais mais comumente afetados. Não há relato de sintomatologia associada com as lesões, sendo descobertos durante exames de rotina (Meleti *et al.,* 2007). A aparência clínica não é diagnóstica da lesão; portanto uma bióspia é necessária para excluir outras lesões pigmentadas tais como mácula melanótica, tatuagem por amálgama e melanoma (Buchner *et al.,* 2004; Meleti *et al.,* 2007). Diferentemente dos melanocíticos normais que se encontram como células isoladas ao longo da camada basal do epitélio, as células névicas tendem a se agrupar formando estruturas conhecidas como tecas (Meleti *et al.,* 2007).

Os NMBs são classificados em 5 tipos: (1) juncional; (2) composto; (3) subepitelial (algumas vezes referido como intramucoso); (4) azul e (5) combinado. No tipo juncional, ilhas de células névicas são organizadas ao longo da junção do epitélio com o tecido conjuntivo. O nevo composto é caracterizado pela presença de parte das células névicas ao longo da junção epitélio/conjuntivo e outra parte somente no tecido conjuntivo, enquanto que no nevo subepitelial as células encontram-se em sua totalidade no tecido conjuntivo. O nevo azul é caracterizado pela proliferação subepitelial de células pigmentadas e alongadas, algumas vezes associado com resposta fibrótica estromal e presença de melanófagos. O nevo combinado demonstra uma combinação de nevo azul com outro tipo de nevo, normalmente nevo composto (Buchner *et al.,* 2004; Meleti *et al.,* 2007).

Ácido graxo sintase (FASN)

Ácido graxo sintase (FASN) é a enzima metabólica responsável pela síntese endógena de ácidos graxos saturados de cadeia longa, a partir dos substratos acetil-CoA e malonil-CoA (Stoops & Wakil, 1981; Tsukamoto *et al*., 1983; Kuhajda *et al.,* 2000; Chirala *et al.,* 2001; Ragan *et al.,* 2001; Baron *et al*.,

2004). Estruturalmente, FASN é um homodímero formado por duas cadeias polipeptídeas longas com massa molecular de aproximadamente 270 kDa e meia-vida de 12,2 h (Graner *et al.,* 2004).

Os ácidos graxos produzidos participam na composição das membranas celulares atuando como hormônios ou mensageiros intracelulares e são formas de armazenamento de energia no organismo (Chirala *et al.,* 2003; Kumar-Sinha *et al.,* 2003). Além disso, os produtos sintetizados pela FASN, especificamente o palmitato e estearato, servem como substrato para construção dos esfingolipídeos, ceramidas e glicolipídeos necessários à progressão da divisão celular, formação de estruturas cerebrais e funções neurológicas (Chirala *et al.,* 2003).

A expressão de FASN é baixa ou até mesmo ausente em tecidos normais, exceto no fígado, tecido adiposo, mama durante a lactação, endométrio na fase proliferativa e pulmões de recém-nascidos (Kuhajda *et al.,* 2000; Chirala *et al.,* 2001; Kusakabe *et al.,* 2002). A atividade desta enzima também é baixa na maioria dos tecidos normais, exceto os lipogênicos, uma vez que a maior parte dos ácidos graxos usados pelas células provém da dieta (Weiss *et al.,* 1986; Baron *et al.,* 2004; Menendez *et al.,* 2005a).

Medes *et al.* (1953) foram os primeiros autores a relatarem aumento na síntese de ácidos graxos nos tecidos tumorais. Esse achado foi confirmado por Weiss *et al.* (1986), os quais demonstraram ser a biossíntese endógena através do FASN responsável pela maior parte dos ácidos graxos em células malignas, independentemente do suprimento nutricional. Diversos são os tumores que apresentam aumento da atividade desta enzima tais como carcinoma de mama (Milgraum *et al.,* 1997), de ovário (Alo *et al.,* 2000), de próstata (Dhanasekaran *et al.,* 2001; Swinnen *et al.,* 2002; Dowling *et al.,* 2009), de endométrio (Pizer *et al.,* 1998), de pulmão (Piyathilake *et al.,* 2000), de cólon (Visca *et al.,* 1999), de esôfago (Nemoto *et al.,* 2001), de estômago (Kusakabe *et al.,* 2002; van de Sande *et al.,* 2005), de bexiga (Visca *et al.,* 2003), carcinoma espinocelular bucal

(Krontiras *et al.,* 1999; Agostini *et al.,* 2004; Silva *et al.,* 2004), melanoma (Innocenzi *et al.,* 2003; Kapur *et al.,* 2005; Carvalho *et al.,* 2008) e sarcomas de tecidos moles (Takahiro *et al.,* 2003; Rossi *et al.,* 2006). Em melanomas, por exemplo, observou-se que a alta expressão de FASN está associada a uma maior taxa de recorrência, maior risco de desenvolvimento de metástase e, conseqüentemente, pior prognóstico (Innocenzi *et al.,* 2003; Kapur *et al.,* 2005). Tais achados são justificados pelo fato de FASN ter grande participação na formação de membranas celulares, uma vez que produz componentes como ácidos graxos e seus derivados (Chirala *et al.,* 2003), os quais também agem como mensageiros intracelulares e como forma armazenadora de energia (Kumar-Sinha *et al.,* 2003). Desse modo, sua alta atividade proporciona vantagens para o rápido crescimento celular em neoplasias (Baron *et al.,* 2004).

Ciclo celular

Ciclo celular é definido como uma série de eventos coordenados através dos quais a célula duplica seu conteúdo e se divide. Tradicionalmente é dividido em 4 fases: G1, S, G2 e M. As fases G1, G2 e S em conjunto são chamadas de intérfase, sendo G1 e G2 fases de intervalo, que fornecem tempo para a célula crescer e averiguar o meio interno e externo, e S, a fase de síntese onde ocorre a duplicação do DNA (Malumbres & Barbacid 2001; Li *et al.,* 2006; Hochegger *et al.,* 2008; Alberts *et al.,* 2004). A fase M corresponde à mitose, quando os cromossomos são separados e as células divididas (citocinese) (Nurse, 1997; Cooper & Hausman, 2009). Depois que a citocinese é completada, a nova célula gerada pode continuar a divisão celular ou interromper sua proliferação. Células que escolhem a última opção entram em um estado conhecido como “quiescência” ou G0, no qual parâmetros bioquímicos permanecem pobremente definidos. As células que continuam a proliferar avançam para a fase G1 de um novo ciclo (Malumbres & Barbacid, 2005).

Controle do ciclo celular

Para assegurar a correta progressão do ciclo celular, as células apresentam uma série de pontos de checagem, os quais previnem que a célula entre em uma fase até que tenha completado com sucesso a fase anterior. Desequilíbrios nestes controles resultam em proliferação descontrolada e possibilitam o crescimento neoplásico (Malumbres & Barbacid, 2001). Existem 4 pontos de checagem bem caracterizados, sendo estes modulados por fatores internos e externos. Ao final da fase G1 existe um ponto após o qual a célula esta comprometida com o ciclo, chamado “ponto de restrição”, termo este proposto por Arthur Pardee em 1974. O ponto de restrição em G1 corresponde ao ponto de checagem onde são verificados tamanho e estado fisiológico da célula, bem como as interações com o meio extracelular. Caso haja alguma alteração nesses parâmetros, as células podem interromper a proliferação e/ou entrar em morte por apoptose. O ponto de checagem da fase S averigua possíveis erros na replicação do DNA. Ao final de G2 há busca por DNA danificado ou não duplicado, além de análise da correta duplicação dos centrossomos. Na fase M, o ponto de checagem identifica se os cromossomos foram corretamente alinhados ao fuso mitótico (Malumbres & Barbacid, 2001; Bucher & Britten, 2008).

A progressão pelas fases do ciclo celular é resultado de uma seqüência de ativações e inibições de quinases dependentes de ciclinas (cdks), enzimas que regulam positivamente o ciclo celular, sendo que a interação dessas quinases com suas respectivas ciclinas permite a correta progressão do ciclo em células normais (Malumbres & Barbacid, 2005; Fecher *et al.,* 2009). Por exemplo, para dar início à fase S, é necessária a formação dos complexos ciclina D/cdk4/6 e ciclina E/cdk2, enquanto que, para se progredir pela fase S, é necessária a formação do complexo ciclina A/cdk2 (Hochegger *et al.,* 2008; Fecher *et al.,* 2009).

As ckis são proteínas conhecidas como inibidores de quinases dependentes de ciclinas e controlam a ativação ou inibição de complexos quinases, sendo composta por p16, p21, p27 e p57 (Ellis *et al.,* 1999; Joyce & Harris, 2010). Todas

as ckis resultam em parada do ciclo celular após serem ativadas por estímulos antimitogênicos ou quando superexpressas (Carnero, 2002). Muitos tumores apresentam alteração na atividade de ckis, indicando que estas proteínas são críticas para o controle da proliferação (Ellis *et al.,* 1999).

O aumento da proliferação celular na ausência de estímulos externos é uma característica comum aos tumores malignos, do mesmo modo que distúrbios no controle normal do ciclo celular geram instabilidades genômicas que contribuem para o desenvolvimento e/ou progressão de muitas malignidades (Li *et al.,* 2006; Malumbres & Barbacid, 2009).

Reguladores da transição de G1 para S do ciclo celular

p16

A proteína nuclear supressora de tumor p16 é codificada por 156 aminoácidos através do gene *CDKN2A* localizado no cromossomo 9p21. É uma das proteínas responsáveis por controlar a transição G1-S (Li *et al.,* 2006). O aumento na sua expressão é observado na senescência celular e também no envelhecimento de vários tecidos. Por se ligar especificamente a cdk4 e cdk6, p16 inibe a formação dos complexos ciclina D1/cdk4/6, necessários para a fosforilação de Rb. Desse modo, Rb permanece ligado a E2F bloqueando a proliferação celular (Li *et al.,* 2006; Fecher *et al.,* 2009; Mitra & Fisher 2009). A perda da expressão de p16 tem sido demonstrada em quase 50% dos melanomas primários, com níveis muito similares em muitos outros tumores malignos (Li *et al.,* 2006).

p21

Proteína que contém 21 kDa, com aproximadamente 166 aminoácidos codificados pelo gene *CDKN1A* localizado no cromossomo 6 (6p21.2). Está localizada no núcleo e citoplasma das células, embora somente a forma nuclear da proteína p21 tenha função de cki (Li *et al.,* 2006; Abbas & Dutta, 2009). p21 é ativada pelo supressor de tumor p53 quando há danos no DNA e também age

como supressora de tumor, inibindo as cdks e a fosforilação da proteína Rb (Ho *et al*., 2007; Abbas & Dutta, 2009). p21 está envolvida na senescência celular, diferenciação e apoptose através de mecanismos independentes de p53. Apresenta efeitos positivos e negativos na progressão de G1 para S, com predomínio dos efeitos inibitórios. Quando presente em baixas concentrações, p21 facilita a ligação de ciclina D1 com cdk4/6, porém em altas concentrações inibe a atividade do mesmo complexo (Li *et al.,* 2006). A inibição da progressão do ciclo mediada por p21 ocorre também pela inibição da atividade de cdk2 (Abbas & Dutta, 2009). Desse modo, p21 se liga a uma larga escala de complexos ciclina/cdk, com preferência por aqueles que contêm cdk2 (Li *et al.,* 2006). Redução da expressão de p21 está associada com tumores malignos humanos de diversas localizações, tais como carcinoma colorretal, cervical, de cabeça e pescoço, além de carcinoma de pequenas células de pulmão (Abbas & Dutta, 2009). Mutações no gene que codifica p21 foram detectadas em melanomas, porém não está claro seu envolvimento na gênese deste tumor. Como p21 é um inibidor de cdk, uma redução em sua expressão é esperada para promover a proliferação das células tumorais. Porém em alguns estudos, um aumento na expressão de p21 é encontrado e está associada com a diferenciação do melanoma, padrão de crescimento e supressão metastática (Trotter et al., 1997) Os níveis de p21 normalmente são baixos ou indetectáveis na maioria dos nevos, porém há aumento de sua expressão em melanomas primários e mestastáticos (Li *et al.,* 2006; Abbas & Dutta, 2009). O exato mecanismo que promove o aumento na expressão de p21 em melanomas não está esclarecido, apesar de que existem algumas hipóteses para explicar tal aumento como adaptações dos pontos de checagem, mutações no gene p21 e degração da proteína (Li *et al.,* 2006).

p27

Proteína nuclear e citoplasmática de 22 kDa, com aproximadamente 198 aminoácidos codificados pelo gene *CDKN1B*, localizado no cromossomo 12p12-12p13 (Malumbres & Barbacid, 2009). É considerado um supressor de tumor,

embora somente sua forma nuclear tenha essa função (Polyak *et al.,* 1994; Soos et al., 1996; Matsuda & Ichida, 2006). Apresenta 44% de homologia estrutural com p21na porção N-terminal, agindo de maneira semelhante, por se ligar ao complexo ciclina D/cdk4 e por regular negativamente ciclina E/cdk2 e ciclina A/cdk2 (Li *et al.,* 2006; Hershko, 2008). Os níveis da proteína p27 oscilam durante o ciclo celular, sendo mais elevados em G0/G1 e menores em S (Hershko, 2008). A degradação de p27 ocorre no final da fase G1, através de um processo dependente de Skp2 (*S phase kinase-associated protein* 2), uma ligase da ubiquitina E3, responsável pela ubiquitinação de p27 e que, portanto, permite a passagem de G1 para S (Schrump *et al.,* 1996; Loda *et al.,* 1997; Hershko, 2008; Mitra & Fisher, 2009). O gene que codifica p27 é raramente alterado em tumores malignos, mas o baixo nível de p27 foi associado a um pior prognóstico em tumores de mama, próstata, sarcomas e tumores hematológicos (Ellis *et al.,* 1999; Hershko, 2008). O padrão de expressão de p27 por imunoistoquímica em tumores melanocíticos demonstra que há uma perda progressiva na expressão de p27 na transição de nevo para melanoma primário e metastático (Li *et al.,* 2006).

Ciclina D1

Possui 34 kDa e 295 aminoácidos codificados pelo gene *CCND1*, localizado no cromossomo 11q13. Ciclina D1 é sintetizada no início de G1 e age antecipadamente nesta fase com suas cdks associadas em resposta a estímulos mitogênicos, como por exemplo, a ativação do fator de crescimento Ras (Grossel *et al.* 1999; Li *et al.* 2006; Macleod, 2008). Forma complexos com cdk4/6, o que resulta na fosforilação e inativação de Rb e liberação de E2F, levando à progressão do ciclo celular. Tais eventos facilitam à ativação de ciclina E/cdk2 e ciclina A/cdk2, complexos necessários para a entrada e progressão na fase S (Li *et al.,* 2006). Desse modo, quando não há proliferação, não há formação dos complexos com cdks e, portanto, os níveis das ciclinas caem rapidamente. Ciclina D1 é raramente mutada, mas sua superexpressão confere vantagem de crescimento seletivo e, conseqüentemente, age como um indutor de crescimento

em várias neoplasias malignas (Masamha *et al.,* 2009). Níveis altos e constantes de ciclina D1 foram demonstrados em linhagens celulares de melanomas, bem como em metástases desse tumor (Li *et al.,* 2006; Masamha *et al.,* 2009). Estudos de imunoistoquímica em melanomas humanos demonstram aumento na expressão de ciclina D1 em melanomas cutâneos e uveais (Li *et al.,* 2009). Ao contrário dos melanomas, nevos melanocíticos de pele e melanócitos normais adjacentes a tumores apresentam ausência ou fraca expressão de ciclina D1 (Florenes *et al.,* 2000; Sauter *et al.,* 2002). Seykora *et al.* (2003) observaram que a expressão de ciclina D1 foi 2.7 vezes maior em melanomas de pele comparado a nevos melanocíticos.

Skp2

Skp2 (*S phase kinase-associated protein* 2) possui 45 kDa e é um dos componentes do complexo SCFskp2 ubiquitina ligase (SCF, *Skp1-Cullin1-F-box protein*), que também conta com a proteína acessória cks1 (*cyclin kinase subunit 1*) (Bornstein *et al.,* 2003). Contém uma subunidade de ligação de aproximadamente 40 resíduos, denominado F Box, associado com regiões ricas em leucina (Chiariello & Esposito, 2006). Trata-se de uma proteína essencial para a passagem de G1 para S, uma vez que é responsável pela poliubiquitinação e proteólise dos reguladores do ciclo celular, p21, p27 e p57 (Schrump *et al.,* 1996; Loda *et al.,* 1997; Vodermaier, 2004; Hershko, 2008; Mitra & Fisher, 2009). Skp2 reconhece especificamente p27 fosforilada, predominantemente na fase S do que em G1, diminuindo os níveis desse inibidor de cdk e culminando na passagem para a fase S (Chiariello & Esposito, 2006). Os níveis de Skp2 oscilam durante o ciclo celular, atingindo níveis máximos na fase S (Bornstein *et al.,* 2003), sendo que a diminuição de sua expressão, ao menos em células quiescentes, parece depender de um processo de autoubiquitinação (Vodermaier, 2004). A expressão de Skp2 está implicada na transforamção neoplásica assim como os seus níveis também estão correlacionados com o grau histológico, agressividade clínica e prognóstico em linfomas, carcinoma de células escamosas oral, adenocarcinoma

de próstata, adenocarcinomas de ovário, sarcomas de tecido mole, carcinomas gástricos, câncer de mama e sarcoma de Kaposi (Latres *et al.,* 2001; Lim *et al.,* 2002; Gstaiger *et al.,* 2001; Kudo *et al*., 2001; Drobnjak *et al*., 2003; Ben-Izhak *et al.,* 2003; Shigemasa *et al.,* 2003; Oliveira *et al.,* 2003). Estudos demonstram uma relação inversa entre os níveis de Skp2 e p27 em vários tumores, incluindo melanoma de pele onde o aumento da expressão de Skp2 foi verificado durante os diferentes estágios de progressão do tumor (Li *et al.,* 2006).

Marcadores de proliferação celular

Mcm2 e geminina são novos marcadores de proliferação celular bastante promissores estudados em trabalhos recentes. Mcm (proteínas mantenedoras de minicromossomos) é uma família composta por dez proteínas altamente conservadas em eucariotos. As proteínas Mcm-2, Mcm-3, Mcm-4, Mcm-5, Mcm-6 e Mcm-7 se unem formando um complexo hexamérico que tem como função permitir o início da replicação do DNA celular, depois de ativado por diferentes fatores. Geminina é uma proteína estritamente ligada a este processo, pois regula e interage com os fatores de ativação do complexo Mcm-2-7. Se há silenciamento ou inibição da ação de geminina ocorre mais de uma replicação por ciclo celular. Dessa maneira, há grandes chances de amplificação de oncogenes, aumento de instabilidades cromossômicas por acúmulo de mutações e, por conseguinte, elevação do risco de desenvolvimento de fenótipo maligno (Vargas *et al.,* 2008; Gouvêa *et al.,* 2010; Tamura *et al.,* 2010). Os poucos trabalhos que correlacionam estas proteínas a melanomas cutâneos mostram que há aumento de expressão em tumores mais agressivos, tanto clinicamente quanto microscopicamente (Boyd *et al.,* 2008).

O presente estudo terá como objetivo avaliar a expressão imunoistoquímica de ácido graxo sintase (FASN) e de proteínas envolvidas nos mecanismos de controle e progressão do ciclo celular como p16, p21, p27, ciclina D1 e Skp2, além dos marcadores de proliferação celular Mcm-2, Ki-67 e geminina em nevos intramucosos e melanomas primários de boca.

CAPÍTULO 1

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EXPRESSION OF FATTY ACID SYNTHASE (FASN) IN ORAL NEVI AND MELANOMA

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ABSTRACT

OBJECTIVE: The aim of this study was to determine the expression of fatty acid synthase (FASN) in oral nevi and melanomas, comparing the results with correspondent cutaneous lesions. MATERIALS AND METHODS: Expression of FASN was evaluated by immunohistochemistry in 51 oral melanocytic lesions, including 38 intramucosal nevi and 13 primary oral melanomas, and in 10 cutaneous nevi and 14 melanomas. RESULTS: Fatty acid synthase was strongly expressed only in melanomas, either of the oral mucosa or cutaneous. On the other hand most oral and cutaneous nevi were negative, with a few oral cases showing focal and weak expression. CONCLUSION: Fatty acid synthase is expressed in malignant melanocytes, and it can be a helpful marker to distinguish oral melanomas from oral melanocytic nevi.

Keywords: melanocytic nevi, melanoma, fatty acid synthase, immunohistochemistry, mouth.

INTRODUCTION

Fatty acid synthase (FASN) is a multifunctional enzyme that participates in the endogenous synthesis of saturated long-chain fatty acid, from the small carbon precursors acetyl-CoA and malonyl-CoA (Baron *et al*, 2004; Jayakumar *et al*, 1995). FASN is downregulated in most normal cells, except in lipogenic tissues such as liver, sebaceous gland, lactating breast, fetal lung and adipose tissue, because most of the fatty acids are supplied by the diet (Kuhajda *et al*, 2000; Weiss *et al*, 1986). On the other hand, FASN expression is up-regulated in several human cancers including prostate, breast, ovarian, lung, stomach, colon, bladder, oral squamous cell carcinoma, cutaneous melanomas as well as in soft tissue sarcomas (Silva *et al*, 2008, 2004; Carvalho *et al*, 2008; Kusakabe *et al*, 2002; Swinnen *et al*, 2002; Rossi *et al*, 2003; Takahiro *et al*, 2003; Kapur *et al*, 2005) FASN overexpression has also been suggested as a potential prognostic factor associated with a poor prognosis, increased risk of recurrence and metastases (Gansler *et al*, 1997; Shurbaji *et al*, 1996; Innocenzi *et al*, 2003).

Over 90% of melanomas occur in the skin, but they may also affect less frequently the oral mucosa, esophagus, meninges and the eyes (Femiano *et al*, 2008; Prasad *et al*, 2004). In cutaneous melanomas, FASN protein expression has been associated with Breslow thickness, and consequently with a poorer prognosis (Innocenzi *et al*, 2003; Kapur *et al*, 2005). Nevertheless there are no data about the expression of FASN in oral nevi and melanoma.

The objective of this work was to determine the expression of FASN in oral nevi and melanomas, comparing the results with correspondent cutaneous lesions.

MATERIALS AND METHODS

Formalin-fixed, paraffin-embedded tissue blocks were obtained from 51 and 24 oral and cutaneous melanocytic lesions respectively. Oral lesions corresponded to 38 intramucosal nevi and 13 primary oral melanomas, and cutaneous included 10 compound melanocytic nevi and 14 melanomas (11 superficial spreading

melanomas and 3 nodular). All lesions were revised using H&E preparations to confirm the diagnosis. Primary oral melanomas were classified according to Prasad *et al* (2004) and 11 and 2 cases corresponded to level III (very deep invasion), and *in situ* respectively.

Cutaneous melanomas were histologically classified using Breslow thickness and Clark level index (Breslow *et al,* 1970). Five cases had a Breslow thickness < 1 mm, 6 cases between 1 and 2 mm, and 3 cases > 2 mm. One case had Clark level I, 10 cases level II and 3 cases level III.

For immunohistochemical staining, 3µm thick sections mounted on silane-coated glass slides were used. Briefly, the sections were deparaffinized, rehydrated in graded ethanol solutions and after antigen retrieval with EDTA/Tris buffer (pH 9.0) in a microwave oven (1380 W; Panasonic, Brazil), endogenous peroxidase activity was blocked with 20% H_2O_2 by 5 cycles of 5 minutes each. Overnight incubation with the primary antibody anti-FASN (BD Biosciences) diluted in BSA (bovine serum albumin-1:200) was followed by the secondary antibody conjugated with polymer dextran marked with peroxidase (Dako EnVision Labelled Polymer; Dako, Glostrup, Denmark). The reaction was developed with Permanent Red (Permanent Red Substrate System, Dako) and counterstained with Carazzi hematoxylin. Positive and negative controls were included in all reactions.

Fatty acid synthase was analyzed using a combined scoring system based on both the fraction of positive tumor cells and the predominant staining intensity in the tumor according to Innocenzi *et al* (2003). The fraction of positive cells was estimated using a four-tiered scale (< 10% = 1, 11-50% = 2, 51-80% = 3, > 80% = 4). The staining intensity was graded subjectively from 0 to 3 (negative: 0, low intensity: 1, moderate: 2, and strong: 3).

RESULTS

The demographic data of the 75 oral and cutaneous melanocytic lesions are summarized in Table 1. All oral melanomas either *in situ* or invasive showed strong

staining intensity for FASN antibody in more than 80% of malignant cells, while 30 cases of oral melanocytic nevi were negative and 8 expressed the protein focally (<10%) with low intensity. Epithelium of the normal mucosa was negative on the basal layer, and diffusely positive in the cytoplasm of cells of the stratum spinosum and granulosum (Figure 1).

Considering the cutaneous lesions, FASN protein was strongly expressed in more than 80% of neoplastic cells in all cases of melanomas, and negative in all compound melanocytic nevi (Figure 2). In melanomas, staining was more intense in cases with Clark level III and Breslow thickness > 2 mm (3 cases with strong staining) when compared with melanomas Clark levels I and II and Breslow thickness < 2 mm (11 cases with moderate staining). Normal skin epithelium was negative for FASN, except the sebaceous gland which strongly expressed this protein (Figure 2).

DISCUSSION

Fatty acid synthase is the key enzyme responsible for the synthesis of fatty acids, catalyzing the conversion of acetyl-CoA and malonyl-CoA into long-chain fatty acids (Baron *et al*, 2004; Jayakumar *et al*, 1995). Normal cells do not express FASN because they use fatty acids from dietary lipids, except those that are lipogenic (Kusakabe *et al*, 2000). Nevertheless it is abnormally expressed in many human cancers cells because of their increased energy need (da Silva *et al*, 2009; Visca *et al*, 2003).

The regulation of FASN production in normal and malignant cells is complex and not well understood. Progesterone stimulates FASN expression in breast cancer cells lines (Lacasa *et al*, 2001), while in prostate cancer, FASN activity is up-regulated by androgens and epidermal growth factor (Heemers *et al*, 2001). It seems that FASN is essential for cancer cell survival, as its specific inhibitors as cerulenin, C75 or Orlistat, reduce cell proliferation and stimulate apoptosis (Carvalho *et al*, 2008; Li *et al*, 2001; Zhou *et al*, 2003; Alli *et al*, 2005; Kridel *et al*,

2004). These findings indicate that activation of fatty acid synthesis may be required for high proliferation levels of cancer cells (Silva *et al*, 2004).

There are no data on expression of FASN in oral melanocytic lesions, and only two reports consider its relevance in cutaneous melanomas and nevi (Innocenzi *et al*, 2003; Kapur *et al*, 2005). We found that FASN was strongly expressed in all oral melanomas studied, and it was negative or eventually focally and weakly expressed in 8 out of 38 cases of oral nevi. Therefore FASN activation in oral melanomas, as in various other cancers, seems to be relevant for malignant transformation of oral melanocytes. Also, it can be potentially useful as a diagnostic marker to distinguish nevi from melanoma. In fact two cases of *in situ* oral melanomas were also strongly positive for this protein. Surprisingly, Kapur *et al* (2005) demonstrated that congenital nevi show high levels of fatty acid synthase expression, near to that seen for metastatic melanomas. The expression of fatty acid synthase in congenital nevi could represent either persistence of or regression to a fetal immunophenotype, since it is known that fetal cells express high levels of fatty acid synthase (Kusakabe *et al*, 2000). These lesions very rarely occur in the mouth, with no data about FASN expression (Allen and Pellegrini 1995, Rose *et al*, 2003, Meleti *et al*, 2007). There are no evidences that oral melanocytic nevi increase the risk for oral melanomas, but it is accepted that these melanomas are preceded by pigmented lesions of unknown characteristics (Meleti *et al*, 2007). It would be interesting to determine FASN expression in atypical melanocytic hyperplasias of the mouth, but as they are very rare a multicentric study would be necessary to select cases of interest.

Our results with cutaneous nevi and melanomas confirm the data of Innocenzi *et al* (2003) and Kapur *et al* (2005), that are similar to the results found on the oral counterparts, i-e, FASN is strongly positive for melanomas and negative in melanocytic nevi. We also confirmed that increased FASN expression is associated with depth of invasion in cutaneous melanomas, and this differed from the results in oral mucosa, where invasive and *in situ* lesions showed similar strong

positivity. In cutaneous melanomas the highest FASN staining intensity was seen in cases with Clark level III and Breslow thickness > 2 mm. These data are in accordance with Kapur *et al* (2005) who demonstrated that the immunohistochemical intensity of FASN is associated with Breslow thickness, suggesting this protein as a molecular prognostic marker. Innocenzi *et al* (2003) showed that patients with cutaneous melanomas which expressed high levels of FASN had an increased risk of developing metastases and recurrence of the disease. They also demonstrated that melanomas thicker than 2 mm, with a strong FASN immunoreactivity were more likely to be lethal than those with comparatively lower expression. Nevertheless our results suggest that these cutaneous parameters in relation to FASN, may not be applied to oral melanomas. In fact oral melanomas are very aggressive, most with very poor prognosis (Prasad *et al*, 2002).

Considering normal tissues, in the skin the sebaceous glands are strongly positive for FASN, serving as an internal positive control reflecting the active synthesis of fatty acids. On the other hand, the normal oral epithelium was positive for FASN in the strata granulosum and spinosum and negative in the stratum basale. Those results are consistent with the fact that the keratinocytes of the strata granulosum and spinosum contain about three times more lipids than those of the stratum basale (Uchiyama *et al*, 2000), suggesting that fatty acid synthesis is increased during normal oral epithelium differentiation (Silva *et al*, 2008; Uchiyama *et al*, 2000).

In summary, our findings show that oral melanomas express high levels of fatty acid synthase similarly to their cutaneous counterparts. Nevertheless FASN staining intensity in cutaneous melanomas increases with increasing depth of invasion, while in oral melanomas expression seems to be similar. As FASN is practically negative for melanocytic nevi, it can be a useful marker for differential diagnoses of oral and cutaneous benign and malignant melanocytic lesions. Future multicentric studies considering FASN expression in atypical melanocytic lesions of

the mouth would be interesting to better understand the biology and development of oral melanomas.

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TABLE

Table 1. Demographic data of 75 oral and cutaneous melanocytic lesions

Tumor type	**Number of cases**	**Age range (mean years)**	**Male**	**Female**	**Localization**
Intramucosal nevi	38	16-67 (36)	8	30	Hard palate (13), buccal mucosa (11), gingiva (10), not specified (4)
Primary oral melanoma	13	23-86 (58)	4	9	Hard palate (8), hard palate and upper gingiva (3), upper gingiva (2)
Compound nevi	10	18-58 (38)	6	4	Upper extremity (3), lower extremity (2), head and neck (1), trunk (3), not specified (1).
Cutaneous melanoma	14	31-75 (58)	3	11	Upper extremity (3), lower extremity (2), head and neck (4), trunk (5)

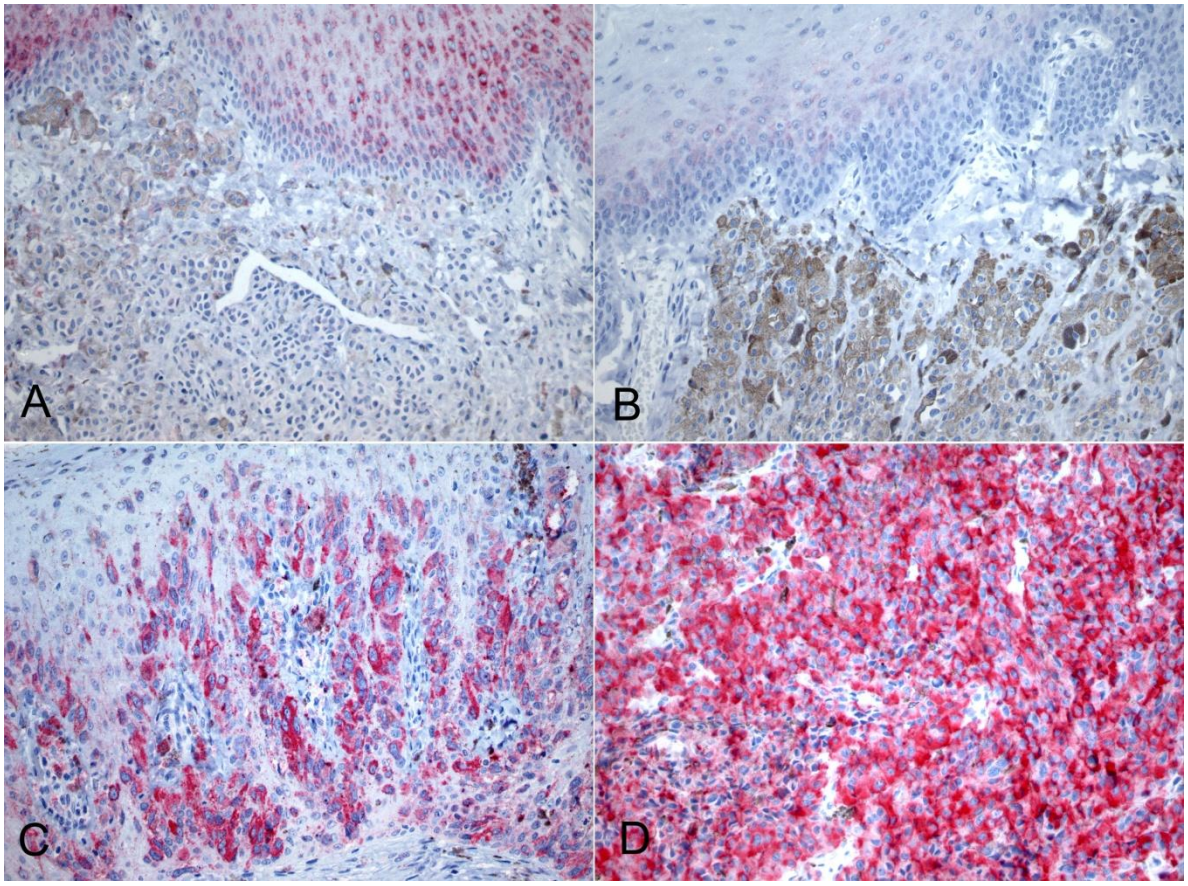

Figure 1. Expression of fatty acid synthase (FASN) in oral melanocytic lesions: (**A)** intramucosal nevi showing FASN expression in the stratum spinosum of normal oral epithelium, while the nevi is negative, **(B)** intramucosal nevi rich in melanin showing absence of FASN expression, **(C)** oral *in situ* melanoma showing positivity for FASN, **(D)** invasive oral melanoma demonstrating high expression of FASN (Envision- Permanent red, **A-D,** x200).

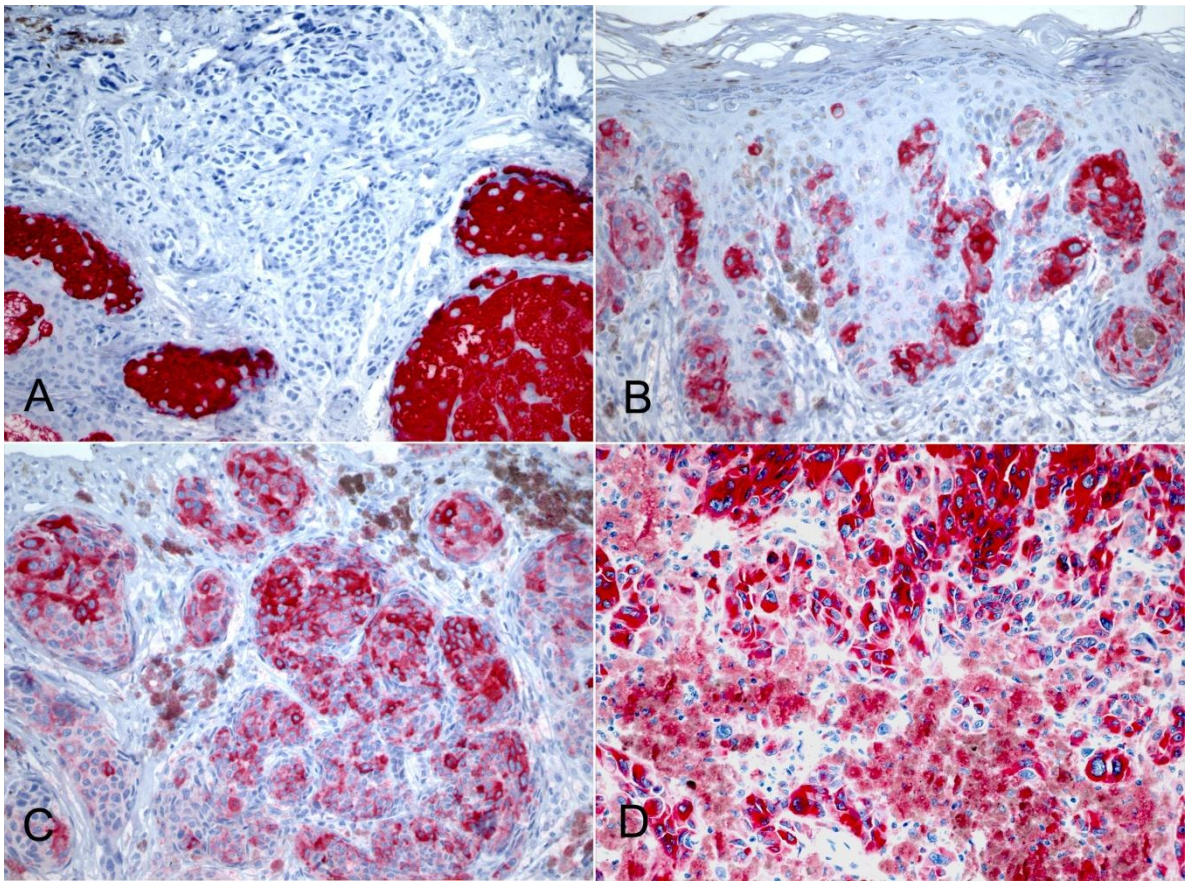

Figure 2. Expression of fatty acid synthase (FASN) in cutaneous melanocytic lesions: **(A)** compound melanocytic nevi which demonstrates strong FASN positivity only in normal sebaceous glands, **(B)** expression of FASN in Clark I melanoma cells, **(C)** Clark II melanoma with melanocytic cells positive for FASN, **(D)** strong FASN expression in neoplastic cells of Clark III melanoma (Envision-Permanent red, **A-D,** x200).

CAPÍTULO 2

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IMMUNOHISTOCHEMICAL EXPRESSION OF p16, p21, p27 AND CYCLIN D1 IN ORAL NEVI AND MELANOMA

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ABSTRACT

The acquisition of abnormalities at G1/S is considered a crucial step in the genesis and progression of melanoma. The expression of cell cycle regulators has also been used in various neoplasms as an adjunct to diagnosis. The aim of this study was to compare the expression of p16, p21, p27 and cyclin D1 in oral nevi and melanomas. Expression of these cell cycle regulatory proteins was evaluated by immunohistochemistry in 51 oral melanocytic lesions, including 38 intramucosal nevi and 13 primary oral melanomas. p16 and p27 were highly expressed in intramucosal nevi, whereas p21 and cyclin D1 expression was higher in oral melanomas. The results indicate that p21 and cyclin D1 may be involved in the development of oral melanomas, and eventually they may be useful in the differential diagnoses of oral benign and malignant melanocytic lesions.

Keywords: oral melanoma; oral nevi; immunohistochemistry; cell cycle.

INTRODUCTION

Oral melanoma is extremely rare, accounting for only 0.2-0.8% of all melanomas, and compared to its cutaneous counterpart, little is known about its pathogenesis, including the etiological factors and molecular mechanisms involved [1]. Melanoma and other cancers, in general, undergo a continuous development from benign to malignant states, as shown in the multistep transition of cutaneous nevi to melanoma [2, 3]. Although most primary oral melanomas appear as new lesions from apparently normal mucosa, about 30-50% are preceded by oral pigmentation for several months or even years. The transformation of melanocytes to melanoma cells involves abnormal cell proliferation associated with alterations in the cell cycle regulatory mechanisms, such as inactivation of cyclin-dependent kinases (cdk) [4, 5].

Among the cell cycle regulators, some studies have focused on the expression of p16, p21, p27 and cyclin D1 in cutaneous melanocytic lesions and melanomas, but little is known of their expression in oral melanoma and there are no data about oral nevi [1, 6, 7]. These proteins are involved in the retinoblastoma protein (pRb) and p53 pathways. p16 protein inhibits cyclin D-cdk4/6 complexes, acting as a tumor suppressor [1]. p21 facilitates the attachment of cyclin D1 to cdk4/6 kinases, which results in the inactivation of pRb and progression through the cell cycle [8]. p27 is a universal cdk inhibitor, which suppresses G1/S cell cycle progression [9]. Cyclin D1 is an important positive regulator of the G1/S cell cycle transition, contributing to the phosphorylation and inactivation of pRb, blocking its growth inhibitory activity and promoting the release of bound E2F, leading to cell cycle progression [10]. Therefore, the objective of this study was to determine by immunohistochemistry the expression of p16, p21, p27 and cyclin D1 in oral melanocytic nevi and melanoma.

MATERIALS AND METHODS

Formalin-fixed, paraffin-embedded tissue blocks were obtained from 51 oral melanocytic lesions, corresponding to 38 intramucosal nevi and 13 primary oral melanomas. All lesions were assessed using H&E preparations to confirm the diagnosis. Intramucosal nevi patients included 30 women and eight men, aged 16 to 67 years, while the primary oral melanomas corresponded to nine women and four men, aged 23 to 86 years. Diagnosis of melanoma was confirmed by clinical and histological characteristics, excluding the presence of melanoma at other anatomical sites and consequently the possibility of oral metastasis. Melanomas were histologically classified according to Prasad et al. [11]; 11 and two cases corresponded to level III (very deep invasion) and *in situ*, respectively.

For immunohistochemical staining, 3 µm thick sections mounted on silane-coated glass slides were used. Briefly, the sections were deparaffinized and rehydrated in graded ethanol solutions. After antigen retrieval with EDTA/Tris buffer (pH 9.0) in a microwave oven (1380 W; Panasonic, São Paulo, Brazil), endogenous peroxidase activity was blocked with 20% H_2O_2 using five cycles of 5 minutes each. Overnight incubation with the primary antibodies for p16, p21, p27 and cyclin D1 (Table 1) diluted in BSA (bovine serum albumin) was followed by incubation with the secondary antibody conjugated with polymer dextran marked with peroxidase (Dako EnVision Labeled Polymer; Dako, Glostrup, Denmark). Reactions were developed with a solution containing 0.6 mg/ml 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma, St. Louis, MO, USA) and 0.01% H_2O_2 and counterstained with Carazzi's hematoxylin. Positive and negative controls were included in all reactions. Only nuclear staining was considered positive. As we used DAB as the developer, the reactions could be confounded by melanin in the cytoplasm of benign and malignant melanocytes, but this was not a problem for interpretation as we used only nuclear markers. Immunohistochemical results were quantified considering the percentage of cells expressing nuclear positivity, counting 1000 cells per slide. Immunoreactive cells were counted

randomly with a minimum of 10 high-power fields (x400) according to Stefanaki et al. [6]. The staining intensity was subjectively classified as weak (+), moderate (++) or strong (+++).

RESULTS

The immunohistochemical results of the 51 oral melanocytic lesions are listed in Tables 2 and 3. p16 was strongly expressed in more than 70% of cells in all cases of intramucosal nevi, while only one oral melanoma case was positive in 35% of neoplastic cells, mainly in the central part of the tumor (Fig. 1). p21 was negative in all intramucosal nevi, but epithelial cells of the normal oral mucosa were positive in the stratum spinosum, serving as an internal positive control. On the other hand, p21 was expressed in all oral melanomas investigated, showing weak to moderate nuclear expression in 10% to 50% of tumor cells, mainly on the superficial central compartment of the tumor rather than the tumor margins (Fig. 2). p27 was strongly expressed in all nevi, with more than 65% of cells positive. Six out of 13 cases of oral melanomas were positive for p27, but with only 5% to 20% of the cells being positive, weakly or moderately. The labeling was heterogeneous, not involving preferential areas (Fig. 3). Cyclin D1 was negative in all nevi, and only normal epithelial cells of the stratum spinosum were positive. All oral melanomas showed cyclin D1 positivity in 10% to 40% of the tumor cells, mainly in the invasive tumor component, with moderate to strong staining intensity (Fig. 4).

DISCUSSION

Melanoma is known to exhibit aberrant expression of cell cycle-regulating genes, and these abnormalities at the G1/S checkpoint are considered crucial steps in the genesis and progression of melanoma [12, 13]. In this study, we evaluated the immunohistochemical expression of the cell cycle regulators p16, p21, p27 and cyclin D1 in 38 intramucosal nevi and 13 primary oral melanomas. There are no data on the immunohistochemical expression of these proteins in intramucosal nevi and only two reports considering p16 expression in oral melanomas [1, 14].

The negative cell cycle regulators p16 and p27 were diffusely expressed at high levels in all intramucosal nevi cells, in contrast to oral melanomas, which demonstrated a much lower expression of these proteins. In agreement with these results, cutaneous nevi have been shown to be uniformly positive for p16 in more than 70% of cells [7, 15]. On the other hand, loss of p16 expression has also been demonstrated in almost 50% of primary cutaneous and oral melanomas, similarly to many other cancers, including pancreatic, esophageal, lung, head and neck, breast, bladder, brain and ovarian [1, 5, 13, 14]. Although there is general consensus regarding the loss of p16 expression in cutaneous melanomas, it is suggested that this occurs in later stages of the disease, as it is not altered in melanoma *in situ* [16]. However, these findings may not be applied to oral melanomas, because we found reduced p16 expression also in oral *in situ* melanomas, suggesting that this alteration may be relevant in the initial phases of oral melanoma development. In oral nevi, expression of p16 was not altered, indicating that in the mouth, nevi do not precede melanoma formation [5]. Reduced expression of p16 in cutaneous melanomas is also associated with a significant risk of recurrence and it is considered an independent predictor for decreased patient survival [13, 17, 18].

p27 was strongly expressed in all nevi cells, in contrast to oral melanomas where six out of 13 cases were weakly or moderately positive. High expression of p27 is a feature of benign lesions, and it can be used to distinguish benign and malignant tumors [5]. Strong p27 nuclear staining has been shown in cutaneous nevi, including Spitz nevi [9, 19], and its expression is progressively lost during the development of melanomas [9, 13, 19]. There are few studies reporting the association of p27 with clinical outcome in melanoma patients. Flørenes et al. [20] found that decreased expression of p27 was significantly associated with increasing Breslow thickness and reduced disease-free survival in primary nodular melanomas. However, other authors have reported that p27 nuclear staining was not associated with patient prognosis [19].

p21 is a cdk inhibitor, so its decreased expression is expected to increase cell proliferation. However, only a few studies have shown that loss of p21 may increase tumorigenic potential in melanocytic lesions [21, 22]. In agreement with our results, p21 levels are usually low or undetectable in the majority of cutaneous nevi, with greater expression in primary melanomas [8, 21, 22]. The exact mechanism causing the increased expression of p21 in primary melanomas is unclear, although possible hypotheses include microenvironmental signals, checkpoint adaptation, p21 mutations, altered or inhibited p21 binding to cyclin/cdk complexes and abnormal protein degradation [5]. On the other hand, the expression of p21 is lower in melanoma metastases when compared with the corresponding primary lesions in most studies [20, 22]. This indicates that downregulation of p21 expression in melanomas is associated with invasiveness and development of a metastatic phenotype. The prognostic value of p21 in melanoma patients is unclear. A significant correlation between protein expression and tumor thickness has been reported in some studies, with thick tumors expressing significantly lower levels of p21 protein, supporting the hypothesis that loss of p21 function may be associated with invasiveness and a metastatic phenotype [21, 22].

Oral nevi were negative for cyclin D1, whereas all oral melanomas studied were positive in 10–40% of the malignant cells. These data are in accord with literature reports of negative or weak expression of cyclin D1 in benign melanocytic lesions and high levels in cutaneous melanomas [22-25]. Besides cutaneous melanomas, increased expression of cyclin D1 has been reported in uveal melanomas, and now we also confirm in oral melanomas, suggesting that it has an oncogenic role in the pathogenesis of this aggressive neoplasm [13, 24, 26]. Some studies have also shown that cyclin D1 is associated with unfavorable outcomes in cutaneous melanomas and is an independent risk factor for the development of metastases [25, 26].

It is important to note that oral melanoma is a rare lesion usually diagnosed at advanced stages, and compared to its cutaneous counterpart some differences are observed. In fact oral melanomas differ from cutaneous in several aspects, including risk factors as actinic radiation, a family history and association with atypical nevi, factors applied mainly to cutaneous melanoma [27]. Genetic events are also distinct from those of its cutaneous counterpart. Mutation and up-regulation of c-KIT has been identified in both oral and cutaneous melanomas which may activate downstream molecules such as RAS and RAF, leading to tremendous cell proliferation [28]. BRAF mutations are classical events in cutaneous melanomas, present in two thirds of these melanomas, while in oral melanoma these mutations are very rare [28, 29]. Those factors in association with our results make oral melanoma unique from cutaneous melanomas.

In summary, our findings show that p16 and p27 were diffusely expressed at high levels in all intramucosal nevi cells in contrast to oral melanomas, which showed overexpression of p21 and cyclin D1. These cell cycle regulators may be involved in the pathogenesis of oral melanomas and may eventually be useful markers for differential diagnoses of oral benign and malignant melanocytic lesions. Primary oral melanoma is a rare lesion. This explains the small number melanoma cases in our series, but future multicentric studies would be useful to better understand the biology and development of oral melanomas.

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Table 1. Antibodies used for immunohistochemical evaluation of 38 intramucosal nevi and 13 primary oral melanomas

Antibody	Clone	Dilution	Source
p16	SC-1661	1:100	Santa Cruz*
p21	WAF1/CIP1	1:50	Dako®**
p27	SX53G8	1:100	Dako®**
Cyclin D1	DCS-G	1:100	Dako®**

* Santa Cruz, Santa Cruz, California, USA.

** Dako, Dako Corporation, Carpinteria, California, USA.

Table 2. Characteristics of intramucosal nevi and immunohistochemical findings

Case	Sex	Age (years)	Location	p16	p21	p27	Cyclin D1
01	F	25	Hard palate	70%	0	65%	0
02	F	35	Buccal mucosa	80%	0	80%	0
03	F	41	Not specified	75%	0	75%	0
04	M	16	Hard palate	70%	0	80%	0
05	F	32	Buccal mucosa	85%	0	85%	0
06	M	26	Buccal mucosa	70%	0	75%	0
07	F	27	Hard palate	70%	0	80%	0
08	F	45	Buccal mucosa	80%	0	65%	0
09	F	Not specified	Hard palate	75%	0	85%	0
10	F	36	Gingiva	80%	0	90%	0
11	M	23	Hard palate	80%	0	85%	0
12	M	29	Not specified	70%	0	80%	0
13	F	67	Buccal mucosa	80%	0	65%	0
14	F	49	Buccal mucosa	90%	0	80%	0
15	F	25	Hard palate	75%	0	75%	0
16	M	28	Not specified	80%	0	70%	0
17	F	26	Gingiva	75%	0	90%	0
18	M	34	Buccal mucosa	80%	0	65%	0
19	F	28	Hard palate	70%	0	75%	0
20	F	40	Gingiva	75%	0	80%	0

Case	Sex	Age (years)	Location	p16	p21	p27	Cyclin D1
21	F	33	Buccal mucosa	70%	0	70%	0
22	F	35	Hard palate	80%	0	80%	0
23	F	28	Gingiva	70%	0	75%	0
24	M	37	Hard palate	90%	0	65%	0
25	F	39	Buccal mucosa	80%	0	70%	0
26	F	Not specified	Gingiva	70%	0	70%	0
27	F	28	Hard palate	85%	0	90%	0
28	F	37	Gingiva	85%	0	65%	0
29	F	32	Buccal mucosa	70%	0	80%	0
30	F	31	Hard palate	80%	0	80%	0
31	M	38	Gingiva	80%	0	70%	0
32	F	29	Gingiva	75%	0	80%	0
33	F	30	Hard palate	70%	0	80%	0
34	F	35	Gingiva	85%	0	80%	0
35	F	31	Buccal mucosa	70%	0	75%	0
36	F	29	Hard palate	80%	0	75%	0
37	F	34	Gingiva	75%	0	70%	0
38	F	30	Not specified	85%	0	80%	0

F, female; M, male

Table 3. Characteristics of primary oral melanomas and immunohistochemical findings

Case	Sex	Age (years)	Location	p16	p21	p27	Cyclin D1
1	F	65	Hard palate	0	15%	0	25%
2	F	78	HP and upper gingiva	0	20%	10%	10%
3	M	50	Hard palate	0	10%	0	30%
4	F	23	Upper gingiva	0	15%	5%	10%
5	F	43	Hard palate	0	10%	0	20%
6	M	38	Hard palate	0	10%	10%	30%
7	F	55	Hard palate	0	30%	0	35%
8	F	86	HP and upper gingiva	35%	50%	20%	40%
9	M	70	Upper gingiva	0	15%	0	30%
10	F	54	Hard palate	0	10%	10%	30%
11	F	76	HP and upper gingiva	0	25%	0	20%
12	M	47	Hard palate	0	15%	10%	10%
13	F	39	Hard palate	0	20%	0	15%

F, female; M, male; HP, hard palate

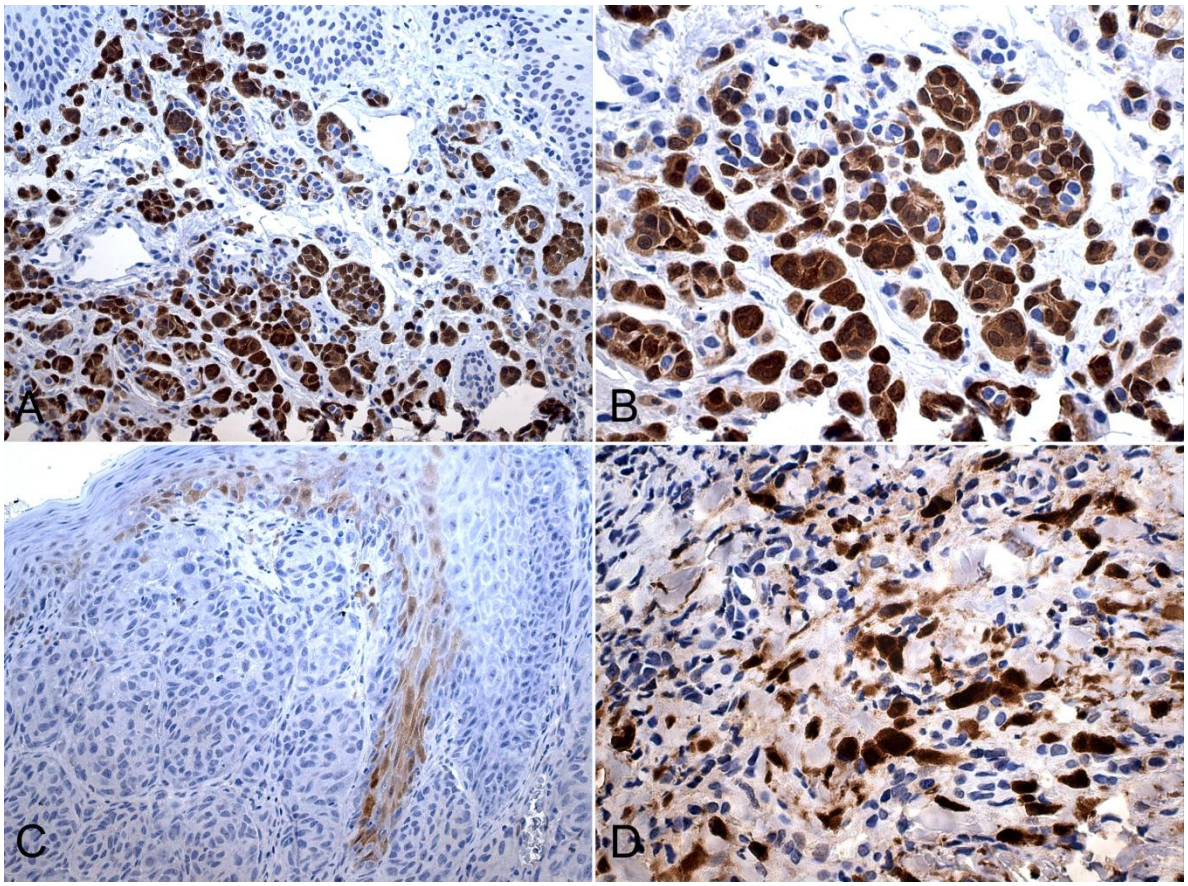

Figure 1. Expression of p16 in oral melanocytic lesions. As we used DAB to develop the reactions, the color in the photographs can be confounded with melanin in the cytoplasm of benign and malignant melanocytes, but this did not interfere with analyzes of the results, as we used only nuclear markers. **(A-B)** intramucosal nevi showing diffuse strong nuclear expression of p16 in melanocytic cells nests, **(C)** malignant cells of invasive oral melanoma negative for p16. Note that cells of the stratum spinosum of normal oral epithelium were positive, serving as an internal positive control, **(D)** one case out of 13 cases of melanoma expressed nuclear p16 in the neoplastic cells, as shown here (immunoperoxidase, **A,C,** x200; **,B,D** x400).

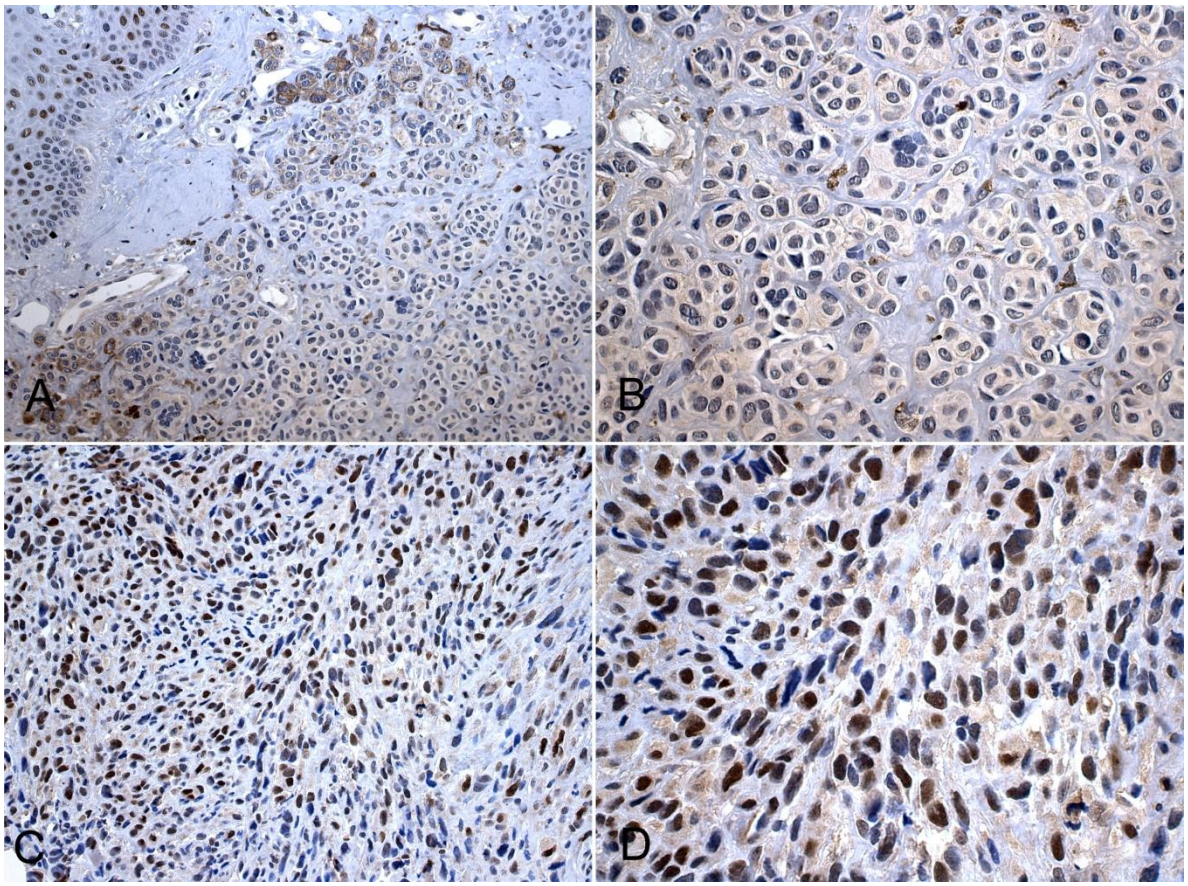

Figure 2. Expression of p21 in oral melanocytic lesions: **(A)** intramucosal nevi showing expression of nuclear p21 only in the epithelial cells of the normal oral mucosa, serving as internal control, **(B)** melanocytes of intramucosal nevi showing absence of p21 nuclear expression, **(C-D)** invasive oral melanoma expressing moderate nuclear positivity for p21 in the tumor cells (immunoperoxidase, **A,C,** x200; **,B,D** x400).

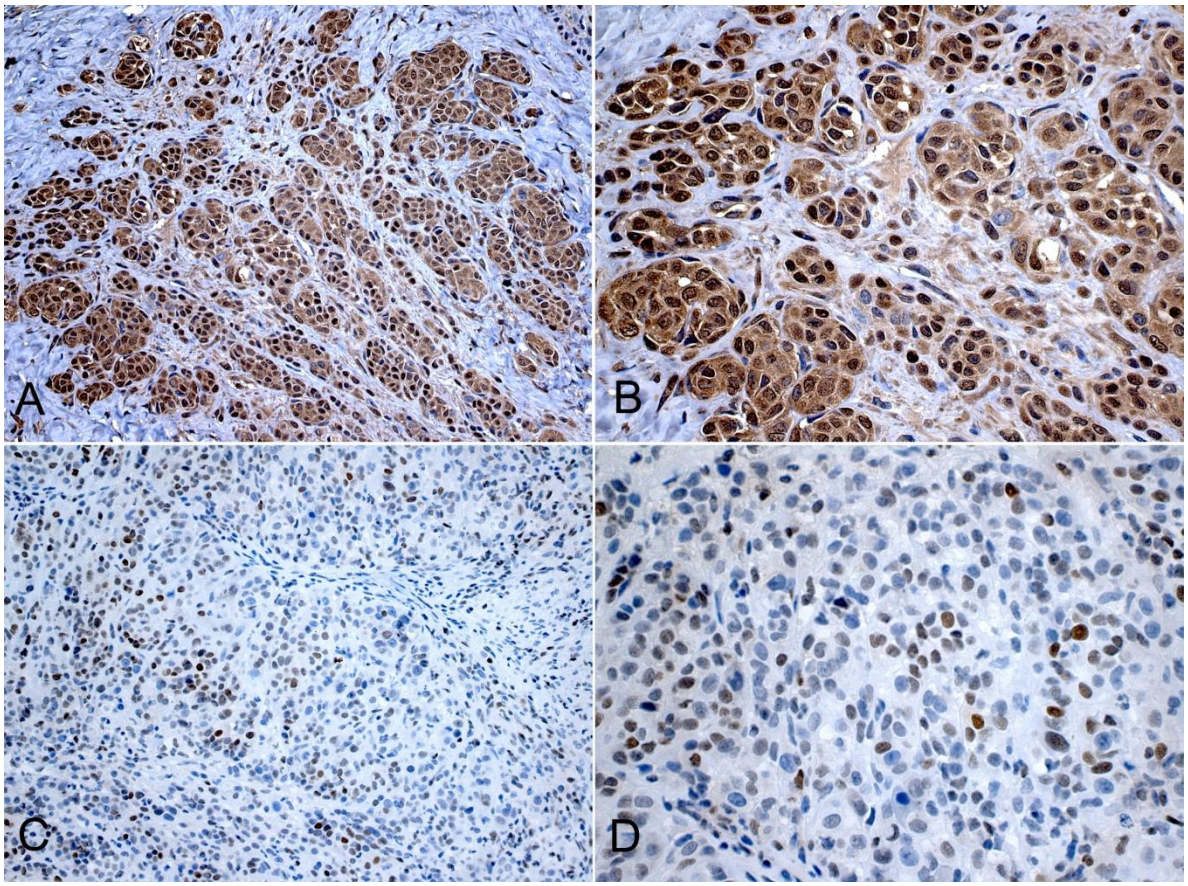

Figure 3. Expression of p27 in oral melanocytic lesions: **(A-B)** intramucosal nevi showing strong p27 expression in the nuclei of melanocytic cell nests, **(C-D)** invasive oral melanoma presenting weak to moderate nuclear expression of p27 in few malignant cells (immunoperoxidase, **A,C,** x200; **,B,D** x400).

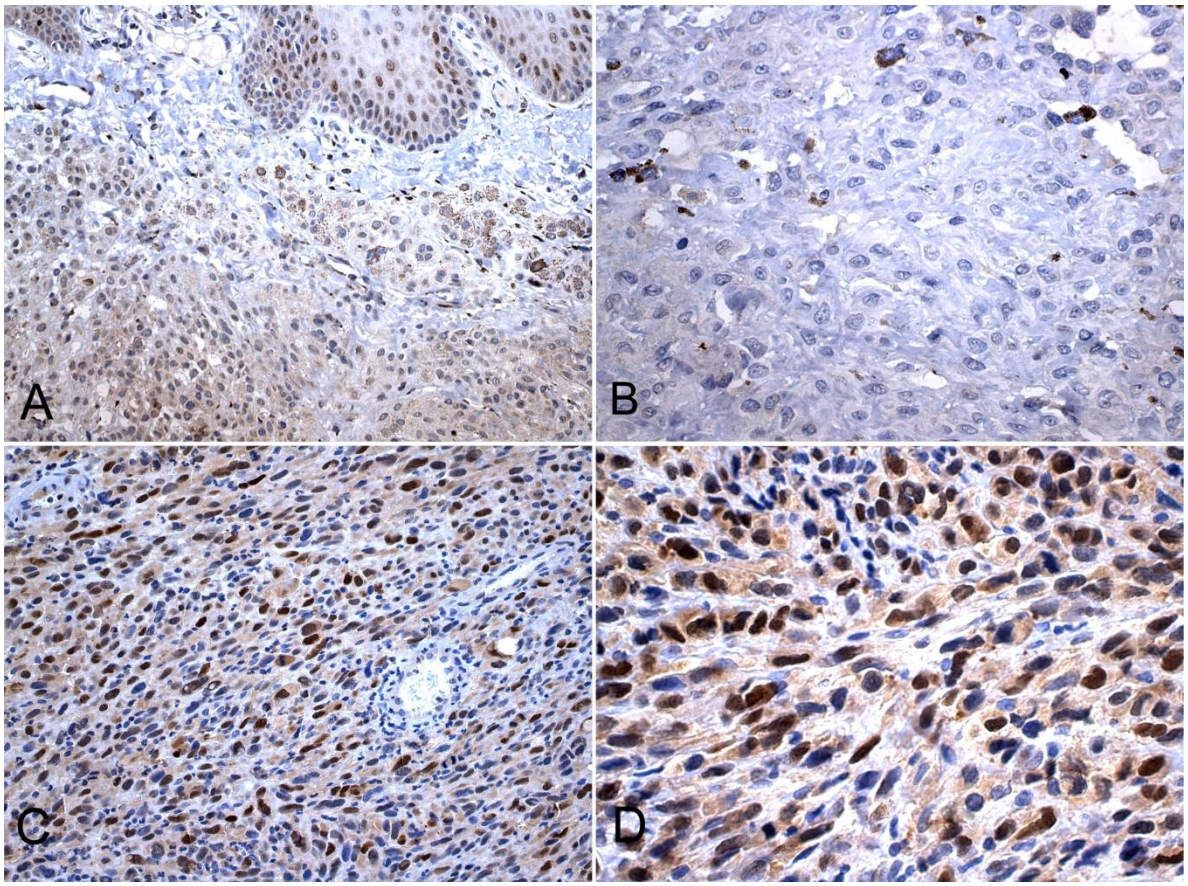

Figure 4. Expression of cyclin D1 in oral melanocytic lesions: **(A)** intramucosal nevi showing nuclear cyclin D1 expression in epithelial cells of the normal oral mucosa, serving as an internal positive control, while the nevi is negative, **(B)** intramucosal nevi showing absence of cyclin D1 nuclear expression, **(C-D)** invasive oral melanoma showing cyclin D1 positivity in neoplastic cells (immunoperoxidase, **A,C,** x200; **,B,D** x400).

CAPÍTULO 3

Artigo publicado na *Annals of Diagnostic Pathology*, 2013 Feb;17(1):32-36.

EXPRESSION OF MINICHROMOSOME MAINTENANCE 2, KI-67 AND GEMININ IN ORAL NEVI AND MELANOMA

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ABSTRACT

Evaluation of cell cycle using antibodies against nuclear proteins involved in regulating DNA replication has gained special interest in the effort to predict biological behavior of benign and malignant tumors. The aim of this study was to analyze the expression of minichromosome maintenance 2, Ki-67 and geminin in oral nevi and melanomas. Expression of these cell proliferation markers was evaluated by immunohistochemistry in 49 oral melanocytic lesions, including 38 intramucosal nevi and 11 primary oral melanomas. The labeling index (LI) of each proliferation marker was assessed considering the percentage of cells expressing nuclear positivity out of the total number of cells, counting 1000 cells per slide. Minichromosome maintenance 2, Ki-67 and geminin were rarely expressed in intramucosal nevi, in contrast to oral melanomas, which showed high levels of these cell proliferation markers, particularly minichromosome maintenance 2, indicating it is a more sensitive marker in primary oral melanomas than Ki-67 and geminin. These results indicate that these markers may be involved in the pathogenesis of oral melanomas and could be eventually useful as an additional diagnostic tool for differential diagnosis of oral benign and malignant melanocytic lesions.

Keywords: oral melanoma; oral nevi; Mcm-2; Ki-67; geminin; immunohistochemistry.

INTRODUCTION

Oral melanoma is a rare lesion, comprising 0.2-0.8% of all melanomas, and contrary to its cutaneous counterpart, little is known about its etiological factors and molecular mechanisms involved. Evaluation of cell cycle proteins by immunohistochemistry has gained special interest in the effort to predict biological behavior and to differentiate benign and malignant tumors [1]. The minichromosome maintenance (Mcm) proteins are essential for the initiation and elongation of DNA replication and comprise six proteins, Mcm-2 to Mcm-7 [2]. They have a fundamental role in DNA replication regulation, and their deregulated expression have been used as a prognostic indicator and also have been considered a novel class of cellular proliferation marker [3, 4]. Ki-67 has largely been used as a tool to estimate the proliferation potential and patient's prognosis [2]. Geminin is thought to be a regulator of the process that inhibits DNA re-replication, by binding Cdt1 and preventing the recruitment of Mcm complex during S, G2, and early M phases of the cell cycle [4].

Recent studies have shown that Mcm proteins and geminin could be sensitive proliferation markers, and may serve as novel biomarkers to detect pre-malignant and malignant lesions [3-5]. The expression of Mcm proteins and geminin has not yet been studied in oral benign and malignant melanocytic lesions. Therefore, the objective of this study was to determine by immunohistochemistry the expression of the cell proliferation markers Mcm-2, Ki-67 and geminin in oral melanocytic nevi and melanoma.

MATERIAL AND METHODS

Formalin-fixed, paraffin-embedded tissue blocks were obtained from 49 oral melanocytic lesions, corresponding to 38 intramucosal nevi and 11 primary oral melanomas. All lesions were revised using H&E preparations to confirm the diagnosis. Intramucosal nevi patients included 30 women and 8 men, aged 16 to 67 years, located in hard palate (n = 13), buccal mucosa (n = 11), gingiva (n = 10), and not specified (n = 4) while the primary oral melanomas corresponded to 8

women and 3 men, aged 23 to 86 years, located in hard palate (n = 6), hard palate and upper gingiva (n = 3), and upper gingiva (n = 2). Diagnosis of melanoma was confirmed by clinical and histological characteristics, excluding the presence of melanoma at other anatomical sites and consequently the possibility of oral metastasis. Melanomas were histologically classified according to Prasad et al. [6] and all cases corresponded to level III (very deep invasion).

For immunohistochemical staining, 3 µm thick sections mounted on silane-coated glass slides were used. Briefly, the sections were deparaffinized and rehydrated in graded ethanol solutions. After antigen retrieval with EDTA/Tris buffer (pH 9.0) in a microwave oven (1380 W; Panasonic, São Paulo, Brazil), endogenous peroxidase activity was blocked with 20% H_2O_2 using five cycles of 5 minutes each. Overnight incubation with the primary antibodies for Mcm-2, Ki-67 and geminin (Table 1) diluted in BSA (bovine serum albumin) was followed by incubation with the secondary antibody conjugated with polymer dextran marked with peroxidase (Dako EnVision Labeled Polymer; Dako, Glostrup, Denmark). Reactions were developed with a solution containing 0.6 mg/ml 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma, St. Louis, MO, USA) and 0.01% H_2O_2 and counterstained with Carazzi's hematoxylin. Positive and negative controls were included in all reactions. Only nuclear staining was considered positive. As we used DAB as the developer, the reactions could be confounded by melanin in the cytoplasm of benign and malignant melanocytes, but this was not a problem for interpretation as we used only nuclear markers.

The labeling index (LI) of each proliferation marker was assessed considering the percentage of cells expressing nuclear positivity out of the total number of cells, counting 1000 cells per slide. The slides were examined under a Leica DMR microscope and images were captured using a Leica digital camera (Leica Microsystems Inc., 1700 Leider Lane, Buffalo Grove, IL, USA). Immunoreactive cells were counted randomly with a minimum of 10 high-power

fields (x400), with the help of an image computer analyzer (ImageJ, Image Processing and Analysis in Java).

RESULTS

The epithelial cells of the normal oral mucosa showed a similar pattern of immunostaining for Mcm-2, Ki-67 and geminin, with positivity restricted to the nuclei of basal epithelial cells, together with some cells in the immediate suprabasal layers, serving as an internal positive control. The most superficial cells of the epithelium were negative in all cases. Mcm-2, Ki-67 and geminin were rarely expressed in intramucosal nevi, with LI lower than 1% in all cases. The location of expression of these markers was heterogeneous, not involving preferential areas of nevus cells nests. (Figures 1, 2 and 3).

The LI of Mcm-2, Ki-67 and geminin in primary oral melanomas were 42.5% (range: 15.5% to 65%), 31.7% (range: 10.3% to 52.7%), and 10.5% (range: 6.8% to 17.3%) respectively. Although the expression of Mcm-2 was higher than Ki-67 and geminin, the pattern was similar for the three markers, with nuclear immunoreactivity diffusely spread in the tumor cells (Figures 1, 2 and 3).

DISCUSSION

Melanoma is known to exhibit aberrant expression of proliferation markers and these abnormalities are considered important steps in the genesis and progression of melanoma [4]. In this study, we evaluated the immunohistochemical expression of the proliferation markers Mcm-2, Ki-67 and geminin in 38 intramucosal nevi and 13 primary oral melanomas. There are few studies about expression of Mcm protein and geminin in oral neoplasias, and to the best of our knowledge, there are no data about the immunohistochemical expression of these proteins in intramucosal nevi and primary oral melanomas [2, 7, 8].

Minichromosome maintenance 2, Ki-67 and geminin were rarely expressed in intramucosal nevi, with LI lower than 1% in all cases. These data are in accordance with literature reports of low or undetectable expression of proliferation

markers in benign cutaneous melanocytic lesions, confirming the benign biologic behavior of these lesions [5]. The immunohistochemical expression of Mcm-2, Ki-67 and geminin in normal oral stratified epithelium restricted to the basal and parabasal compartments, which are common sites of proliferative cells, was useful as an internal positive control [1].

It is interesting that there was a high expression of Mcm-2 in primary oral melanomas. The LI of Mcm-2 expression was consistently higher than Ki-67 and geminin, all presenting a similar staining pattern, indicating that Mcm-2 may be a sensitive proliferation marker of oral melanomas. This has also been shown recently in other tumors as renal cell carcinoma, hepatocellular carcinoma, prostate, bladder and breast cancer, as well as in oral squamous cell carcinoma and salivary gland tumors, i-e the proportion of cells expressing Mcm-2 is consistently higher than those expressing Ki-67 or geminin [2, 4, 9-13].

It is important to compare our results in oral melanocytic lesions with their cutaneous counterpart. In agreement with our results, Boyd et al. [5] also demonstrated significant differences of Mcm-2 expression between benign nevi and cutaneous melanoma. They showed high expression of Mcm-2 in primary and metastatic cutaneous melanoma with mean LI of 49.1% and 40.9% respectively, while the percentage of positively staining nuclei in benign cutaneous nevi were only 1.2%. These results may suggest that Mcm-2 appears to differ significantly in melanocytic neoplasms and potentially provides an additional tool for distinguishing benign tumors from their malignant counterparts [5].

The differences found in LI of Mcm-2, Ki-67 and geminin in primary oral melanoma cases may be explained by the different expression of these markers in the cell cycle phases. It has been shown that expression of Ki-67 varies in the G1 phase, being present from late G1 to mitotic phase, while Mcm-2 is expressed in the early G1 phase and through the whole cell cycle [14]. Thus, the expression interval is obviously shorter for Ki-67 than for Mcm-2, which might result in identification of cells in the cell cycle and also non-cycling cells G0, with

proliferative potential. Nevertheless, some studies showed that Mcm positive and Ki-67 negative cells have been characterized as non-proliferative, licensed cells, such as oocytes and pre-menopausal breast cells [15, 16]. Geminin is a negative regulator of DNA replication by preventing Cdt1 from loading Mcm proteins onto DNA, and it is only expressed from S to M phases [2]. Therefore, it is possible that a proportion of melanocytes cells are in a primed replication licensed state characterized by Mcm-2 expression and absence of Ki-67 and geminin, as shown in epithelial tumors studies [2, 17]. Therefore, similar to data described for other tumors, our study suggests that Mcm-2 is a more sensitive proliferation marker in primary oral melanomas compared to Ki-67 and geminin.

The present study did not consider the relation between expression levels of these proliferations markers with prognosis of oral melanoma, but it is well known that oral melanoma have a poor prognosis. In most cancer high proliferative indexes are usually related to a poorer prognosis [9-13,18]. Nevertheless, some authors demonstrated that high geminin LI is a significant predictor of better prognosis in some tumors like oral squamous cell carcinoma, rectal cancer and high-grade astrocytic brain tumors. This was explained by the higher chemo-radiosensitivity of geminin positive cells, during the G2 or M-phase [4, 19, 20].

In summary our findings show that Mcm-2, Ki-67 and geminin were rarely expressed in intramucosal nevi. In contrast, oral melanomas showed high levels of these cell proliferations markers, and Mcm-2 may be a more sensitive marker compared to Ki-67 and geminin. These markers may be involved in the pathogenesis of oral melanomas, and could be eventually useful as an additional diagnostic tool for differential diagnoses of oral benign and malignant melanocytic lesions. Further evaluation about expression of these proliferative markers may be useful in delineating the biologic behavior of these tumors and warrants additional research.

ACKNOWLEDGEMENTS

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TABLE

Table 1. Antibodies used for immunohistochemical evaluation of 38 intramucosal nevi and 11 primary oral melanomas

Antibody	Clone	Dilution	Source
Mcm-2	CRCT2.1	1:40	Novocastra*
Ki-67	MIB-1	1:100	Dako®**
Geminin	EM6	1:50	Novocastra*

* Novocastra Laboratories, UK.

** Dako, Dako Corporation, Carpinteria, California, USA.

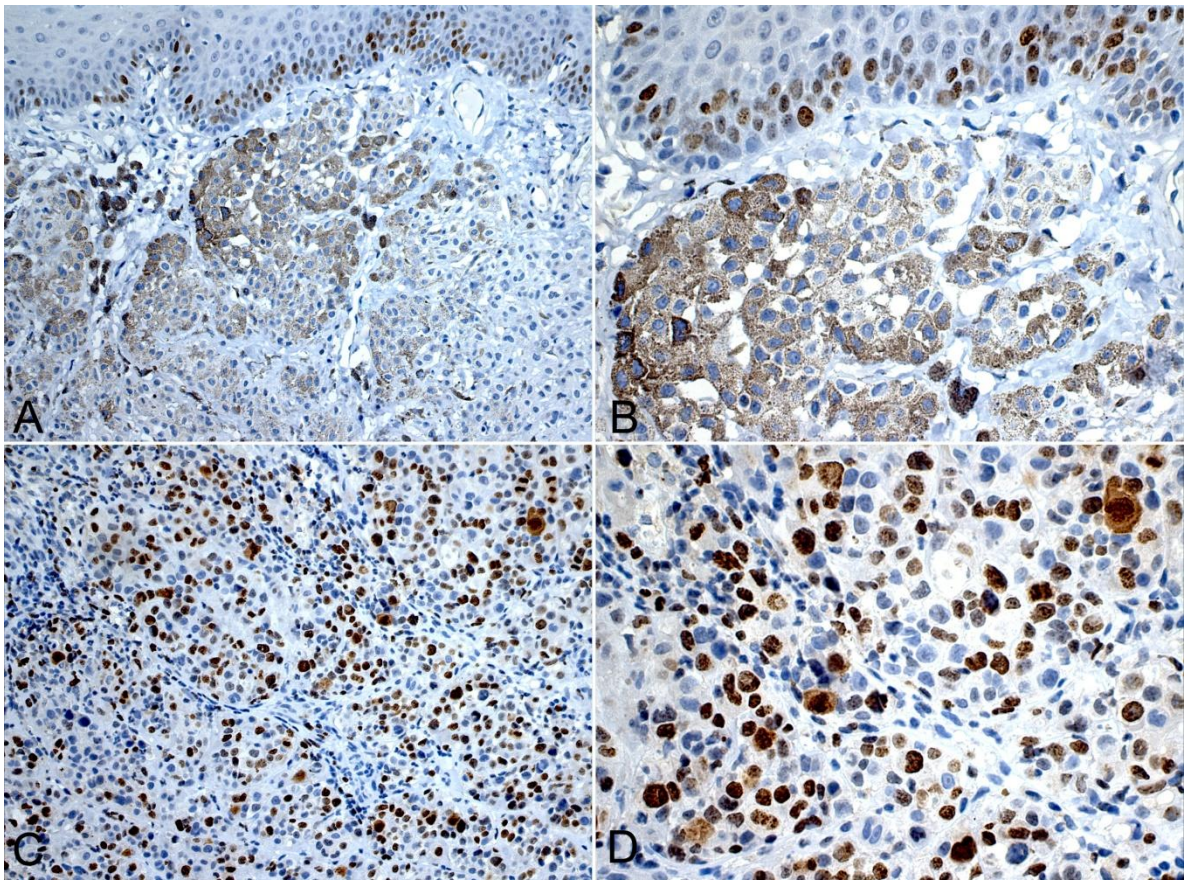

Figure 1. Expression of Mcm-2 in oral melanocytic lesions. As it was DAB to develop the reactions, the brown color of the positive reaction, at first can be confounded with melanin in the cytoplasm of benign and malignant melanocytes, but this do not interfere with analyzes of the results, as it was only nuclear markers. **(A-B)** intramucosal nevi showing nuclear expression of Mcm-2 only in basal and suprabasal epithelial cells of the normal oral mucosa, serving as internal control, **(C-D)** invasive oral melanoma showing diffuse Mcm-2 positivity in neoplastic cells (immunoperoxidase, **A,C,** x200; **B,D** x400).

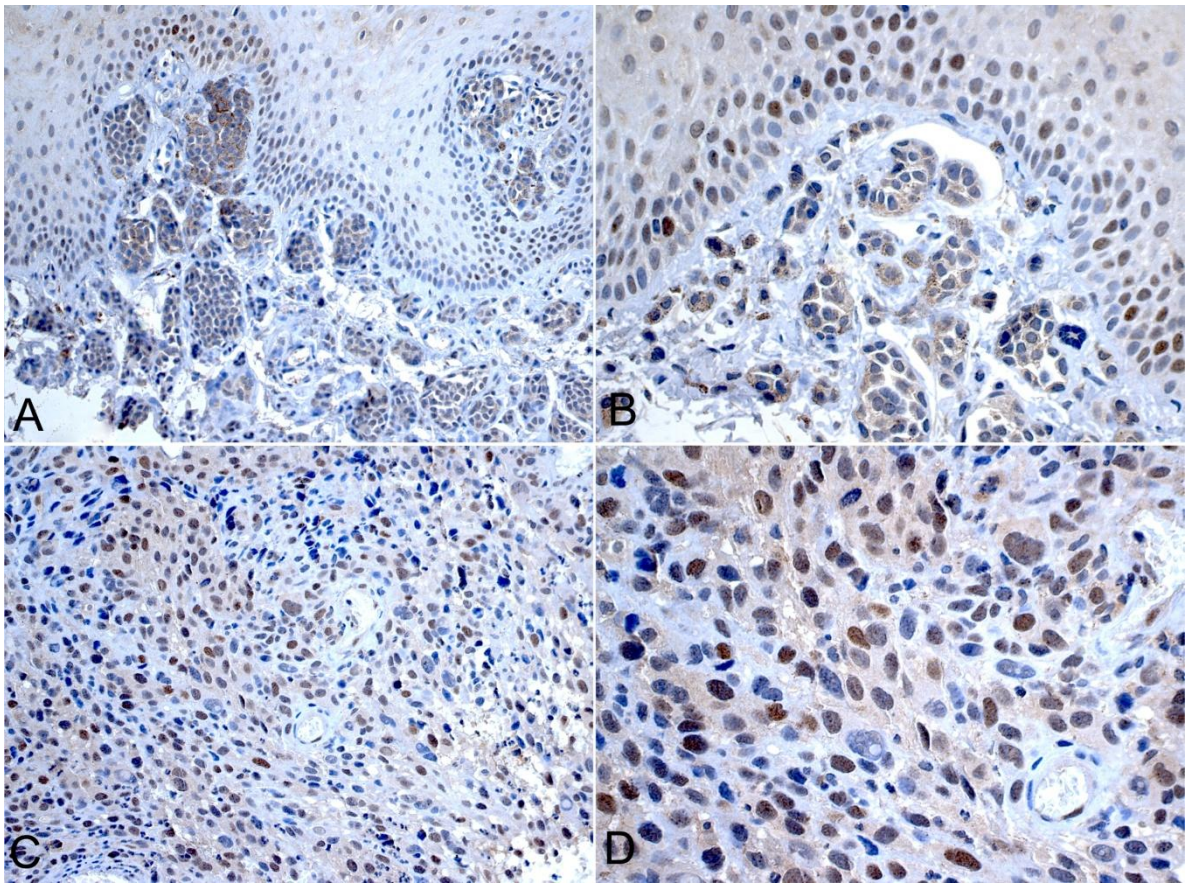

Figure 2. Expression of Ki-67 in oral melanocytic lesions: **(A-B)** intramucosal nevi showing nuclear Ki-67 expression in epithelial cells of the normal oral mucosa, serving as an internal positive control, while the nevi is negative, **(C-D)** invasive oral melanoma expressing nuclear positivity for Ki-67 in the tumor cells (immunoperoxidase, **A,C,** x200; **B,D** x400).

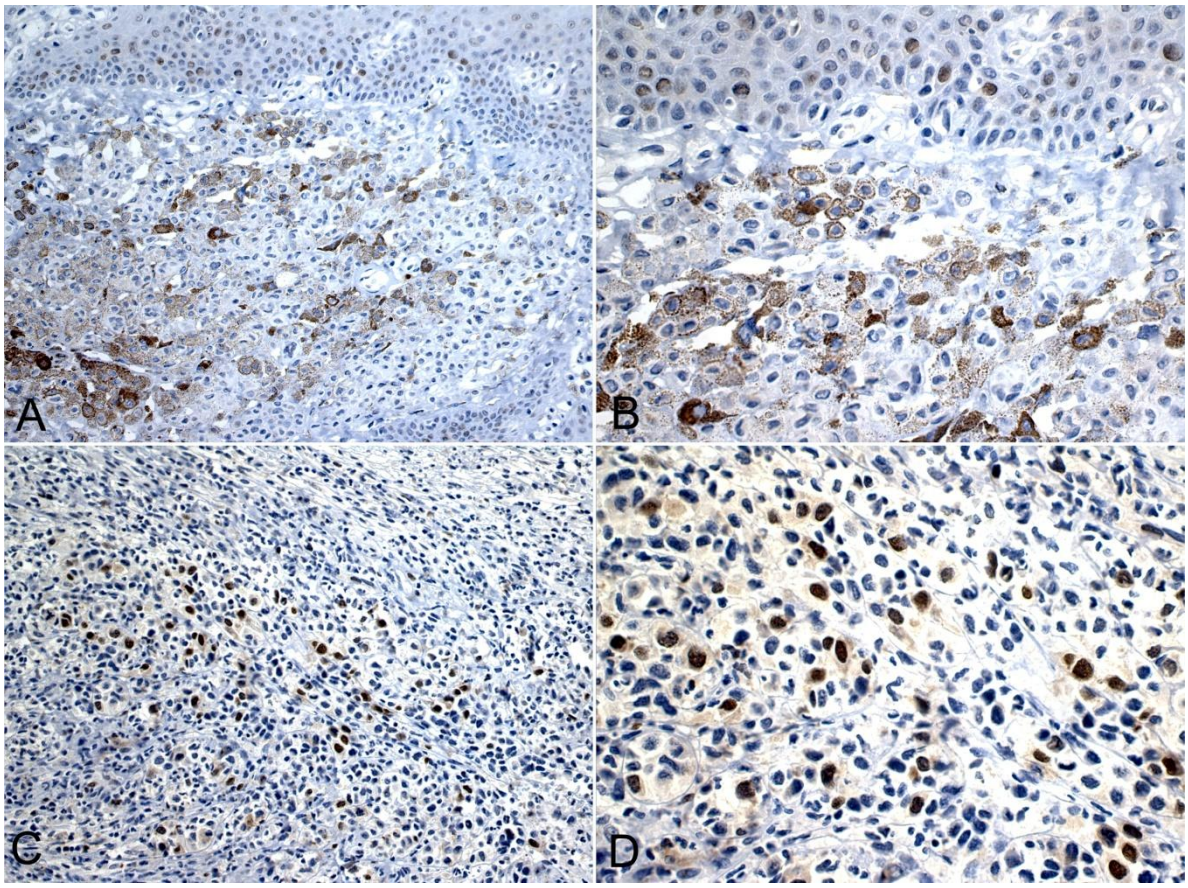

Figure 3. Expression of geminin in oral melanocytic lesions: **(A-B)** melanocytes of intramucosal nevi showing absence of geminin nuclear expression, while the normal oral epithelium is positive in basal and suprabasal layers, **(C-D)** invasive oral melanoma presenting nuclear expression of geminin in malignant cells (immunoperoxidase, **A,C,** x200; **,B,D** x400).

CAPÍTULO 4

Artigo aceito para publicação na *Medicina Oral Patología Oral Cirugia Bucal*.

IMMUNOHISTOCHEMICAL EXPRESSION OF SKP2 PROTEIN IN ORAL NEVI AND MELANOMA

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ABSTRACT

Objective: The aim of this study was to analyze the immunohistochemical expression of Skp2 protein in 38 oral nevi and 11 primary oral melanomas. **Study design:** Expression of this ubiquitin protein was evaluated by immunohistochemistry in 49 oral melanocytic lesions, including 38 intramucosal nevi and 11 primary oral melanomas. The labeling index (LI) was assessed considering the percentage of cells expressing nuclear positivity out of the total number of cells, counting 1000 cells per slide. **Results:** Skp2 protein was rarely expressed in intramucosal nevi, in contrast to oral melanomas, which showed high levels of this protein. **Conclusion:** These results indicate that Skp2 protein may play a role in the development and progression of oral melanomas, and it also could be useful as an immunohistochemical marker for differential diagnosis of oral benign and malignant melanocytic lesions.

Keywords: oral melanoma; oral nevi; Skp2; cell cycle; immunohistochemistry.

INTRODUCTION

The transformation of melanocytes to melanoma cells involves abnormal cell proliferation associated with alterations in the cell cycle regulatory mechanisms (1). Cell cycle progression requires the coordinated performance of a series of regulating molecules that orchestrate cycle transitions through either mitogenic or antiproliferative signals (2). Disruption of the mechanisms involved in protein synthesis and degradation can lead to abnormal cell proliferation and oncogenesis (3). It is well known that the ubiquitin proteasomal pathway plays a paramount role in the degradation of short-lived regulatory proteins involved in the cell cycle (2).

The ubiquitin ligase complex formed by Skp2, Skp1 and cullin F-box (SCF^{SKP2}) is required for direct ubiquination and proteolysis of p27 and other cell cycle regulatory proteins such as cyclin E and the transcription factor E2F-1, performing an S phase promoting function (4,5). Overexpression of Skp2 results in cell cycle progression and eventually neoplastic transformation, as its levels correlates with histologic grade, clinical aggressiveness and prognosis in lymphomas, oral squamous cell carcinoma, prostate adenocarcinoma, ovarian adenocarcinoma, soft tissue sarcomas, gastric carcinomas and breast cancers (6-8).

Recent studies have shown that Skp2 protein expression is implicated in cutaneous melanoma progression, and it may also serve as a biomarker to detect pre-malignant and malignant lesions (3,9). The immunohistochemical expression of Skp2 protein has not yet been studied in oral benign and malignant melanocytic lesions, and therefore, this is the objective of this study.

MATERIALS AND METHODS

Formalin-fixed, paraffin-embedded tissue blocks were obtained from 49 oral melanocytic lesions, corresponding to 38 intramucosal nevi and 11 primary oral melanomas. Intramucosal nevi patients included 30 women and 8 men, aged 16 to 67 years, 13 located in hard palate, 11 in the buccal mucosa, 10 in the gingiva, and

4 the site was not specified. Cases of primary oral melanomas corresponded to 8 women and 3 men, aged 23 to 86 years, 6 located in hard palate, 3 involving the hard palate and upper gingiva, and 2 the upper gingiva. Diagnosis of melanoma was confirmed by clinical and histological characteristics, excluding the presence of melanoma at other anatomical sites and consequently the possibility of oral metastasis. Melanomas were histologically classified according to Prasad et al. (10) and all cases corresponded to level III (very deep invasion).

For immunohistochemical staining, 3 µm thick sections mounted on silane-coated glass slides were used. Briefly, the sections were deparaffinized and rehydrated in graded ethanol solutions. After antigen retrieval with EDTA/Tris buffer (pH 9.0) in a microwave oven (1380 W; Panasonic, São Paulo, Brazil), endogenous peroxidase activity was blocked with 20% H_2O_2 using five cycles of 5 minutes each. Overnight incubation with the primary antibody Skp2 (Santa Cruz Biotechnology, Santa Cruz, California USA) diluted in BSA (bovine serum albumin-1:200) was followed by incubation with the secondary antibody conjugated with polymer dextran marked with peroxidase (Dako EnVision Labeled Polymer; Dako, Glostrup, Denmark). Reactions were developed with a solution containing 0.6 mg/ml 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma, St. Louis, MO, USA) and 0.01% H_2O_2 and counterstained with Carazzi's hematoxylin. Positive and negative controls were included in all reactions. Only nuclear staining was considered positive. As we used DAB as the developer, the reactions could be confounded by melanin in the cytoplasm of benign and malignant melanocytes, but this was not a problem for Skp2 because it is a nuclear marker.

The labeling index (LI) was assessed considering the percentage of cells expressing nuclear positivity out of the total number of cells, counting 1000 cells per slide. The slides were examined under a Leica DMR microscope and images were captured using a Leica digital camera (Leica Microsystems Inc., 1700 Leider Lane, Buffalo Grove, IL, USA). Immunoreactive cells were counted randomly with a

minimum of 10 high-power fields (x400), with the help of an image computer analyzer (ImageJ, Image Processing and Analysis in Java).

RESULTS

The epithelial cells of the normal oral mucosa in nevi and melanomas showed Skp2 protein positivity restricted to the nuclei of basal epithelial cells, together with some cells in the immediate suprabasal layers, serving as an internal positive control. The most superficial cells of the epithelium were negative in all cases (Figure 1). Skp2 protein was rarely expressed in intramucosal nevi, with LI lower than 1% in all cases. The location of Skp2 expression was heterogeneous, not involving specific areas of nevus cells nests (Figure 1). In primary oral melanomas, 17.5% (range: 8.7% to 36.5%) of malignant cells expressed Skp2 in the nucleus, mainly on the superficial central compartment of the tumor rather than in the tumor margins (Figure 2).

DISCUSSION

Melanoma is known to exhibit aberrant expression of cell-cycle-regulating proteins. The F-box protein Skp2 is a component of the ubiquitin protein ligases that play a critical role in the regulation of G to S phase progression (6). Cell cycle progression is driven by an increase of Skp2, which is responsible for ubiquination of some cell cycle proteins such as cyclin E and p27 (4,5). In this study we evaluated the immunohistochemical expression of Skp2 protein in 38 intramucosal nevi and 11 primary oral melanomas, as there are no data on immunohistochemical expression of Skp2 in oral melanocytic lesions and only 4 reports consider its relevance in cutaneous melanocytic lesions (3,9,11,12).

We found low expression of nuclear Skp2 in oral nevi compared to melanomas, confirming an oncogenic potential of Skp2. This is also in accordance with literature reports of negative or weak expression of Skp2 protein in benign cutaneous melanocytic lesions and high levels in melanomas (3,9,11-13). Li et al. (3) reported a progressive and significant increase in the nuclear expression of

Skp2, moving from melanocytic nevi to melanoma *in situ*, primary cutaneous melanoma and metastatic melanoma respectively, suggesting that this protein is implicated in melanoma progression. High levels of Skp2 have also been shown in a variety of cancers such as prostate (14), oral squamous carcinoma (7,15) and colorectal carcinomas (16). Also attesting the importance of Skp2 in tumor development, there is a report using transgenic mouse model where Skp2 overexpression induced prostatic hyperplasia, dysplasia, and low-grade carcinoma (17).

The present study did not consider the relation between expression levels of Skp2 protein with stage and prognosis of oral melanomas, but it is well known that oral melanomas have a poor prognosis and all our cases showed deep invasion. Nevertheless, it is has been shown that Skp2 nuclear protein expression have prognostic impact in some human cancers such as squamous cell carcinoma (7). Nevertheless, in cutaneous melanomas results are controversial, as increased expression of nuclear Skp2 have been correlated either with reduced survival time (9), or that it was not associated with prognosis (3).

In conclusion, our data suggest that increased Skp2 expression plays a role in the development of oral melanomas. Overexpression of Skp2 may represent an important mechanism in abrogating the cell-cycle inhibitory and apoptotic effects of some cell cycle proteins such as of p27 and cyclin E, increasing their degradation, thus contributing to the expansion of malignant clones of melanocytes and tumor progression. Besides to be involved in the pathogenesis of oral melanomas, Skp2 could also be useful as an additional diagnostic marker for differential diagnosis of oral benign and malignant melanocytic lesions.

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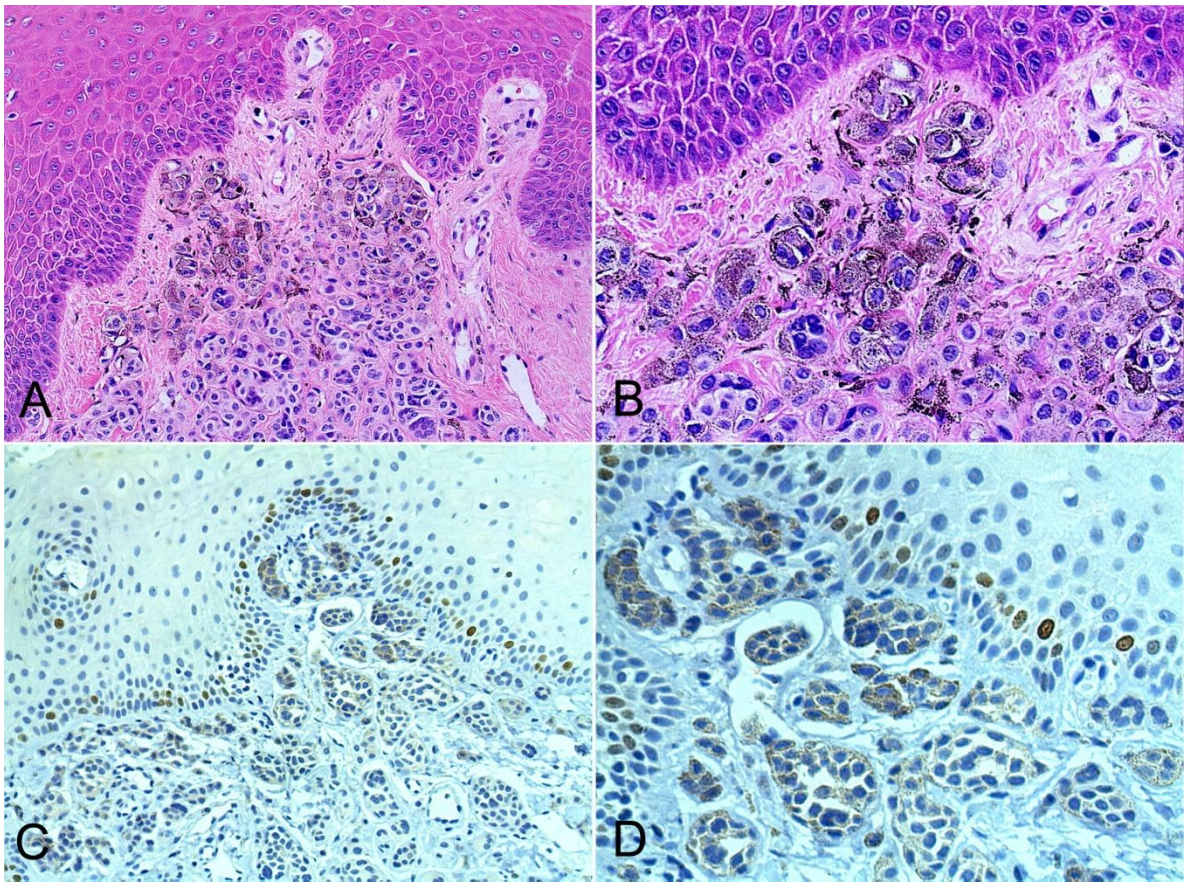

Figure 1. Intramucosal nevi of the gingiva showing sheets and nests of pigmented nevomelanocytes spreading into the underlying connective tissue **(H&E, A- x200, B- x400)**. Intramucosal nevi showing by immunohistochemistry nuclear Skp2 protein expression only in basal and suprabasal cells of the normal oral epithelium, serving as an internal positive control, while the nevic cells are negative **(immunoperoxidase, C- x200, D- x400).**

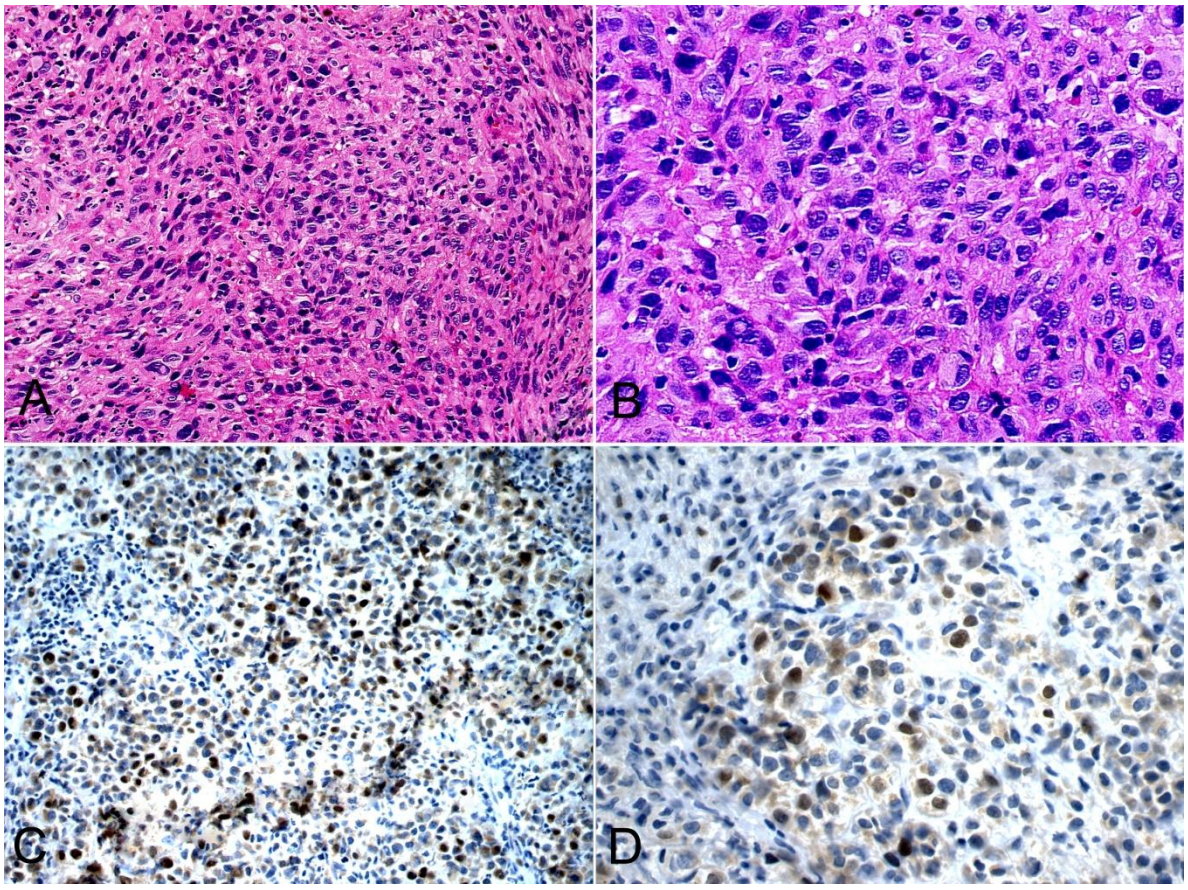

Figure 2. Oral melanoma of the hard palate showing pleomorphic epithelioid neoplastic cells arranged in solid pattern **(H&E, A- x200, B- x400)**. Nuclear expression by immunohistochemistry of Skp2 protein in the tumor cells of oral melanoma **(immunoperoxidase, C- x200, D- x400).**

CONCLUSÃO:

1) Melanomas bucais expressaram altos níveis de ácido graxo sintase (FASN), com intensidade de marcação similar em lesões *in situ* e invasivas. Pelo fato de FASN ter sido praticamente negativo em nevos intramucosos, sugere-se seu uso como um provável marcador para diagnóstico diferencial entre lesões melanocíticas benignas e malignas.

2) p16 e p27 foram altamente e difusamente expressos em todos os casos de nevo intramucoso, enquanto nos casos de melanoma houve superexpressão de p21 e cliclina D1. Esses reguladores do ciclo celular podem estar envolvidos na patogênese do melanoma bucal, podendo eventualmente ser utilizados como marcadores para o diagnóstico diferencial entre lesões melanocíticas benignas e malignas.

3) Mcm-2, Ki-67 e geminina foram raramente expressos nos casos de nevo intramucoso, enquanto nos casos de melanoma houve alta expressão desses marcadores de proliferação celular, particularmente Mcm-2, indicando uma maior sensibilidade desse marcador comparado com Ki-67 e geminina, podendo esse marcador ser utilizado como uma ferramenta adicional para o diagnóstico de melanoma.

4) A alta expressão de Skp2 observada nos casos de melanoma pode representar um mecanismo importante na suspensão dos eventos inibitórios do ciclo celular e degradação de algumas proteínas tais como p27, contribuindo assim para a proliferação de células malignas do melanoma e progressão do tumor. Além de estar envolvida na patogênese do melanoma, Skp2 também pode ser útil como um marcador adicional para o diagnóstico diferencial entre lesões melanocíticas benignas e malignas.

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COMITÊ DE ÉTICA EM PESQUISA
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CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa **"Análise imunoistoquímica da expressão de FASN, p16, p21, p27, ciclina D1, cdk4 e Skp2 em nevos melanocíticos orais e melanomas primários de boca"**, protocolo nº 026/2011, dos pesquisadores Bruno Augusto Benevenuto de Andrade e Oslei Paes de Almeida, satisfaz as exigências do Conselho Nacional de Saúde - Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 25/05/2011.

The Ethics Committee in Research of the School of Dentistry of Piracicaba - State University of Campinas, certify that the project **"Immunohistochemical analysis of expression of FASN, p16, p21, p27, ciclina D1, cdk4 e Skp2 in oral melanocytic nevus and oral primary melanomas"**, register number 026/2011, of Bruno Augusto Benevenuto de Andrade and Oslei Paes de Almeida, comply with the recommendations of the National Health Council - Ministry of Health of Brazil for research in human subjects and therefore was approved by this committee at 05/25/2011.

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