UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

CIRO DANTAS SOARES

ESTUDO COMPARATIVO DA EXPRESSÃO DE p-AKT1, COX-2 E MARCADORES MITOCONDRIAIS EM MELANOMAS CUTÂNEOS E MUCOSOS

COMPARATIVE STUDY OF THE EXPRESSION OF p-AKT1, COX-2 AND MITOCHONDRIAL MARKERS IN CUTANEOUS AND MUCOSAL MELANOMAS

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Tese apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutor em Estomatopatologia, na Área de Patologia.

Thesis presented to the Piracicaba Dental School of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor in Stomatopathology, in Pathology area.

Orientador: Prof. Dr. Jacks Jorge Junior

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RESUMO

Melanomas são neoplasias malignas agressivas que ocorrem principalmente na pele. Os melanomas mucosos de cabeça e pescoço, por outro lado, que ocorrem nas mucosas da cavidade bucal e nasal, são neoplasias malignas extremamente agressivas; e devido sua raridade, estudos mais completos sobre possíveis marcadores prognósticos ainda são escassos na literatura. O presente trabalho foi organizado em 5 capítulos que dissertam sobre a expressão de proteína B quinase fosforilada (p-Akt1), ciclooxigenase 2 (COX-2) e marcadores mitocondriais em uma série de melanomas cutâneos e mucosos de cabeça e pescoço. Como principais resultados, podemos destacar, as conclusões de cada capítulo conforme organizado abaixo. No primeiro capítulo, demonstramos que a proteína p-Akt1 teve maior expressão em melanomas mucosos que nos cutâneos e foi identificada como um fator prognóstico independente em melanomas mucosos. No segundo capítulo, foi estudada a expressão de COX-2 em melanomas orais, demonstrando que essa enzima é um fator prognóstico independente para pacientes com estes tumores. No terceiro capítulo, apresentamos uma série de melanomas amelanóticos e foi possível concluir que a apresentação clínica e microscópica desses tumores pode mimetizar outros tipos de lesões, e que, portanto, podem ser mais difíceis de ser diagnosticados. No quarto capítulo, foi comparada a expressão de COX-2 e de Ki67 em melanomas orais amelanóticos e melanomas orais convencionais. Como principal conclusão, a expressão de COX-2 e o índice de proliferação foram maiores em melanomas orais amelanóticos, indicando provavelmente uma maior agressividade destes tumores. No quinto capítulo, foi avaliada a expressão de três marcadores mitocondriais em melanomas cutâneos e mucosos. Como destaque desse último capítulo podemos citar que a quantidade de mitocôndrias nas células tumorais parece desempenhar um papel importante na patogênese dos melanomas. E que, a proteína de fissão 1 (FIS1) pode ser considerada um fator prognóstico em melanomas orais, enquanto a proteína de fusão 2 (mitofusin 2) está associada com pior prognóstico em pacientes com melanomas cutâneos. Em resumo, os cinco capítulos da presente tese contribuíram para o melhor entendimento dos melanomas mucosos e em conjunto apresentam uma série de possíveis marcadores prognósticos que podem ser estudados e indicados como candidatos para terapias-alvo.

Palavras-chave: Melanomas cutâneos, melanomas mucosos de cabeça e pescoço, p-Akt1, COX-2, marcadores mitocondriais, prognóstico.

ABSTRACT

Melanomas are tumors with an aggressive behavior that occur mainly in the skin. Mucosal head and neck melanomas, on the other hand occur in the mucous membranes of the oral and nasal cavity. These tumors are extremely aggressive; and due to its rarity, studies with these tumors regarding possible prognostic markers are still scarce in the literature. The present PhD thesis was organized in 5 chapters that discuss the expression of phosphorylated serine/threonine-protein kinase 1 (p-Akt1), cyclooxygenase 2 (COX-2) and mitochondrial markers in a series of cutaneous and mucous melanomas of the head and neck. We have highlighted the main conclusions of each chapter, as organized below. In the first chapter, we demonstrated that p-Akt1 protein was overexpressed in mucosal than in cutaneous melanomas and it was identified as an independent prognostic factor for mucosal melanomas. In the second chapter, we have studied the COX-2 expression in oral melanomas, demonstrating that this enzyme is an independent prognostic factor for patients with these tumors. In the third chapter, we present a series of amelanotic melanomas and the main conclusion was: the clinical and microscopic presentation of these tumors may mimic other types of lesions, and therefore they may be more difficult to diagnose. In the fourth chapter, the expression of COX-2 and Ki67 in amelanotic and conventional oral melanomas was compared. As a main conclusion, COX-2 expression was higher in amelanotic oral melanomas, as well as cell proliferation index in these tumors than in conventional oral melanomas. In the fifth chapter, the expression of three mitochondrial markers in cutaneous and mucosal melanomas was evaluated. We have demonstrated that mucosal melanomas have a higher expression of mitochondrial markers than cutaneous ones. Thus, the high number of mitochondria in tumor cells seems to play an important role in the pathogenesis of melanomas. The fission protein 1 (FIS1) was considered a prognostic factor in oral melanomas, whereas mitofusin 2 is associated with worse prognosis in patients with cutaneous melanomas. In summary, the five chapters of this thesis have contributed to a better understanding of mucosal melanomas and together present a series of possible prognostic markers that can be studied and indicated as candidates for target therapies.

Keywords: Cutaneous melanomas, head and neck mucosal melanomas, p-Akt1, COX-2, mitochondrial markers, prognosis.

SUMÁRIO

1 INTRODUÇÃO

Melanomas são neoplasias malignas extremamente agressivas que ocorrem principalmente na pele (Leonardi *et al.*, 2018). Sua etiologia está intrinsicamente ligada a alterações genéticas em células melanocíticas de pacientes com histórico de larga exposição à radiação solar (Curtin *et al.*, 2005). O prognóstico de pacientes com melanoma cutâneo melhorou significativamente com o advento da imunoterapia, um novo tipo de tratamento baseado na reprogramação do sistema imunológico para combater células malignas (Lugowska, Teterycz and Rutkowski, 2017). Ainda assim, alguns casos mais avançados – com metástase à distância, por exemplo – possuem prognóstico ruim (Li *et al.*, 2007).

Os melanomas mucosos de cabeça e pescoço, por outro lado, que ocorrem nas mucosas da cavidade bucal e nasal, são neoplasias malignas extremamente agressivas; e devido sua raridade, estudos mais completos sobre possíveis marcadores prognósticos ainda são escassos na literatura (de Andrade *et al.*, 2012; de-Andrade *et al.*, 2012; Bishop and Olszewski, 2014; Lourenço *et al.*, 2014; Mochel *et al.*, 2015; de Souza do Nascimento *et al.*, 2016). Os fatores etiológicos dos melanomas mucosos de cabeça e pescoço ainda não são conhecidos, o que também dificulta o entendimento da patogenia desses tumores agressivos.

As diferenças biológicas entre melanomas mucosos e cutâneos são objetivo de debate há muito tempo (Luna-Ortiz *et al.*, 2016). No entanto, poucos estudos conseguiram determinar que realmente há uma diferença na biologia tumoral dessas lesões. O pior prognóstico das lesões mucosas quase sempre é associado unicamente ao diagnóstico tardio, que obviamente representa um papel importante, mas talvez não seja o único fator associado com péssimo prognóstico para estes pacientes (Kuk *et al.*, 2016; Moya-Plana *et al.*, 2019).

A biologia tumoral é muito complexa, e envolve vários passos que culminam com a progressão tumoral e o processo metastático. Algumas proteínas desempenham papéis importantes no contexto de migração, invasão e proliferação celular. Dentre elas, a proteína B kinase (também denominada Akt), que é uma serina-treonina com três isoformas bem conhecidas: Akt1, Akt2 e Akt3 (Franks *et al.*, 2016). A isoforma Akt1 é amplamente conhecida por sua atividade no metabolismo celular, ativando vias glicolíticas, apoptóticas e inclusive modulando a atividade de proliferação celular. Esses efeitos têm sido demonstrados em diversos tipos de tumores (Cicenas, 2008; Liu *et al.*, 2010; Agarwal, Brattain and Chowdhury, 2013; Wu *et al.*, 2014). Estudo recente

também demonstrou que a Akt1 desempenha um papel muito relevante no desenvolvimento de metástases de melanoma em um modelo de camundongos (Cho *et al.*, 2015). Em estudo de sequenciamento genético do nosso grupo (dados ainda não publicados), foi possível observar uma diferença na expressão de Akt1 entre melanomas cutâneos e mucosos de cabeça e pescoço.

A ciclooxigenase 2 (COX-2) é uma enzima pró-inflamatória envolvida diretamente no metabolismo do ácido araquidônico (Fitzpatrick, 2005). As consequências da expressão e da atividade de COX-2 nas células tumorais é bem conhecida: ativa vias de angiogênese, facilitando o processo de disseminação tumoral e consequentemente metástases à distância; modulação do processo apoptótico; evasão do sistema imune, e muitas vezes também associado com resistência quimioterápica (Guo *et al.*, 2015; Xia *et al.*, 2015; Hu *et al.*, 2017; Hashemi Goradel *et al.*, 2019). A expressão de COX-2 já foi estudada em melanomas de cavidade oral, e interessantemente, a ausência de COX-2 em nevos orais e cutâneos indica que essa enzima poderia desempenhar um papel central na biologia tumoral dos melanomas (de Souza do Nascimento *et al.*, 2016).

Mitocôndrias são organelas responsáveis pela produção de energia no ambiente intracelular e desempenham um papel crucial durante praticamente todos os processos biológicos. No contexto das células tumorais, vários processos biológicos requerem o ATP como fonte de energia através da fosforilação oxidativa (Corazao-Rozas *et al.*, 2016). Como consequência da fosforilação oxidativa, as mitocôndrias também produzem a maioria das espécies reativas de oxigênio (ERO). As EROs estão implicadas no processo carcinogênico, danificando importantes proteínas e macromoléculas, causando desregulação do ciclo celular e outros processos relevantes para o desenvolvimento e metástase do tumor (Liou and Storz, 2010). Além disso, as mitocôndrias desempenham um papel essencial na apoptose, regulando as vias críticas de sinalização para sua ativação ou inibição (Suen, Norris and Youle, 2008; Srinivasan *et al.*, 2017). Esses eventos ainda não foram estudados em melanomas.

De acordo com o que foi exposto, há uma clara escassez de estudos comparativos com melanomas cutâneos e mucosos de cabeça e pescoço, particularmente, no que se diz respeito a marcadores prognósticos. Na era de terapias-alvo, é sumamente importante entender a biologia tumoral, e assim, aplicar esses conceitos no desenvolvimento de terapias complementares que possam melhorar o prognóstico dos pacientes. Nesse sentido, o objetivo desse estudo foi, avaliar e

comparar a expressão de p- Akt1, COX-2 e marcadores mitocondriais em uma larga série de melanomas cutâneos e mucosos de cabeça e pescoço, bem como validar possíveis marcadores com utilidade prognóstica nestes tumores.

2 ARTIGOS

2.1 Artigo: Phosphorylated Akt1 expression is associated with poor prognosis in cutaneous, oral and sinonasal melanomas

Artigo publicado no periódico Oncotarget
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Abstract

Melanomas are highly aggressive tumours derived from melanocytes, which occur most commonly in the skin. Occasionally, these tumours may appear in oral and sinonasal mucous membranes. In this study, we performed a comparative analysis of the Phosphorylated Akt1 (p-Akt1) expression in 144 patients affected by cutaneous (CM), 34 oral cavity (OM), and 31 sinonasal melanomas (SNM). Similar to the metastatic cutaneous melanomas, p-Akt1 was overexpressed in 17/34 of the oral cavity and 20/31 of the sinonasal melanomas. In addition, the p-Akt1-nuclear expression was associated with poorer cancer-specific survival in cutaneous ($P < .0001$), oral ($P < .0001$), and sinonasal ($P = .001$) melanomas. Multivariate analysis showed p-Akt1 to be an independent prognostic marker in oral ($P = .041$) and sinonasal ($P < .0001$) melanomas patients. In conclusion, p-Akt1 overexpression is an independent prognostic marker in mucosal melanomas and is significantly up-regulated in sinonasal melanomas. As both mucosal and metastatic cutaneous melanomas showed high frequency of p-Akt1 expression, these findings suggest that mucosal melanomas have a biological behaviour, similar to the aggressive cutaneous melanomas.

Keywords: Mucosal melanomas, cutaneous melanomas, prognostic markers, p-Akt1

Introduction

Cutaneous melanomas represent about 1.6% of all cancers, and discreet advances in their treatment have been made over the last decades. Nevertheless, this tumour still remains deathly, especially the metastatic disease [1]. It is estimated that melanomas with lymph nodal metastases are responsible for 59,782 global deaths, with mean overall survival rate of 13% in 5 years [2]. Controlling the advanced-stage disease is the major problem for the treatment of melanomas. Thus, for the development of targeted therapies, making progress in understanding the molecular factors that influence the aggressiveness of the metastatic melanomas is essential.

Until now, comparative studies with melanomas from different anatomic sites that assessed the potential differences and similarities between these tumours are scarce [3–6]. Overall, the etiologic factors and the clinical and biological behaviour of cutaneous melanomas are very distinct from the other melanomas [7, 8]. For example, mucosal melanomas constitute a particular subset of all melanomas characterized by high-aggressive behaviour, tendency to metastasize and consequently association with marked worse prognosis. Sinonasal melanomas in some series have presented a high-mortality index (near 100%) [8, 9]. Clinicopathological parameters determine the prognosis and staging of the melanomas from different anatomic sites [10].

The use of biological markers, especially by immunohistochemistry, for predict prognosis is still poorly explored in mucosal melanomas. The serine/threonine protein B Kinase, known as Akt, has an oncogenic function in several tissues by regulating cell proliferation, migration and invasion [11]. The overexpression of p-Akt1 is correlated with adverse outcome in breast [12], gastric [13] and oesophageal squamous cell carcinomas [14]. In the context of melanoma, an overgrowing interest in determining as p-Akt1 acts during melanoma progression [15, 16] has been recently observed, and its inhibition has emerged as an interesting targeted therapy for these tumours [17].

Although some studies have demonstrated the role of p-Akt1 in several cancers, including melanoma metastases in a mice model [18], no studies have evaluated the prognostic value of the immunohistochemical expression of p-Akt1 in different subsets of melanomas. In the present study, we evaluated the immunohistochemical expression and the prognostic value of p-Akt1 expression in a large cohort of cutaneous, oral, and sinonasal melanomas.

Material and Methods

Ethical issues

The study protocol was approved by the National Commission for Ethics in Research (CONEP-Brazil, CAAE: 72077517.1.0000.5418) and all procedures were in accordance with the Declaration of Helsinki.

Patient samples and data collection

Cutaneous, sinonasal, and oral samples from the surgical samples of patients with melanoma who were referred to four Brazilian and Guatemalan cancer centers since 1997 were collected and further analysed. Melanoma diagnoses were confirmed by three experienced pathologists based on the microscopic examination of haematoxylin–eosin–stained slides, and on the S-100 protein, MART-1/Melan A and gp-100 (HMB-45) expressions.

In total, 209 samples, including cutaneous (n = 144), oral (n = 34) and sinonasal (n = 31) melanomas. The first patient was included in May 1997 and the last patient in February 2016. Surviving patient follow-up was censored on September 10, 2017. Clinical data were collected from the patient's medical records which included age, sex, tumour location, metastases (presence/absence and site), clinical-stage, treatment options, and follow-up data. Tumour stage was determined according to the seventh edition of the staging manual of the American Joint Committee on Cancer [25].

Tissue microarray construction

For construction of the 7 tissue microarray blocks, a previously reported method was followed [41, 54]. Duplicate cores of 2 mm^2 were collected from the original blocks of cutaneous, oral, and sinonasal melanoma patients.

Immunohistochemistry (IHC) and digital scoring

IHC manual technique was performed using 3-mm thick formalin-fixed paraffin-embedded melanomas sections mounted on silane-coated glass slides. An anti-Phospho-Akt1 (clone D7F10, 1:100 dilution; Cell Signalling, Danvers, Massachusetts, USA) was used. The antigen detection was achieved using the ADVANCE™/HRP (code K406889-2; Dako, Carpinteria, CA, USA), revealed with the 3,3′-diaminobenzidine-tetrahydrochloride chromogen and counterstained with Carazzi's haematoxylin.

All slides were scanned into high-resolution images with Aperio Scanscope CS Slide Scanner (Aperio Technologies Inc., Vista, California, USA). In order to calculate the scores of positivity expression of p-Akt1, digital analyses were performed, as previously described [41, 55]. Nuclear p-Akt1 expression was scored based on the

percentage [%] of positive nuclei, assessed digitally with the Nuclear Algorithm (Aperio Technologies Inc.).

Statistical analyses

For the statistical analyses, all collected data were recorded in a password-protected computer database. All statistical analyses were carried out using the SPSS software, version 18.0 (SPSS Inc., Chicago, IL, USA). In the univariate model, the cancer-specific survival (CSS) (defined as the time between the start date of the treatment and the date of death due the disease) and disease-free survival (DFS) (defined as the time between treatment and recurrence) were estimated comparing the Kaplan–Meier survival curves using the log-rank test. The multivariate Cox regression was performed for prognostic significance determination of the p-Akt1 expression Index. For both univariate and multivariate models, the association between the variables age, sex, primary tumour site and p-Akt1 expression and CSS and DFS were evaluated. *p*-value ≤ .05 was considered statistically significant.

Results

Cutaneous melanomas

The age range of the cohort of 144 patients affected by cutaneous melanomas was from 20 to 88 years old at diagnosis (mean age, 56). The three most common sites were the trunk (64/144), followed by the lower limbs (40/144) and the head and neck area (22/144) (Table 1). The median follow-up period was 62 months, ranging from 14 months to 18 years. The 3- and 5-year cancer-specific survival (CSS) rates were 89.9% and 69.3%, respectively. Detailed clinicopathological data from the cutaneous melanomas is shown in Supplementary Table 1.

Table 1: The relationship between clinicopathological characteristics of patients with cutaneous melanomas, p-Akt1 expression and cancer-specific survival

Factors	Sample *n* (%)	CSS (%)		Univariate *P* (log-rank)	Multivariate	
		3-years	5-years		HR (95% CI)	*P*-value
Age						
<56	78	94.8%	87.6%	**.013 (6.118)**	2.029 (.969–4.250)	.061
≥56	66	82.5%	70.4%			
Ulceration						

Factors	Sample *n* (%)	CSS (%)		Univariate *P* (log-rank)	Multivariate	
		3-years	5-years		HR (95% CI)	*P*-value
Present	61	76.5%	60.3%	**<.0001 (39.745)**	1.569 (.499–4.933)	.441
Absent	83	98.8%	94.4%			
Breslow's Thickness						
<1.55	72	100%	98.4%	**<.0001 (42.747)**	10.855 (1.101–107.057)	**.041**
≥1.55	72	78.7%	61.7%			
Mitotic rate						
<3	60	98.3%	94.2%	**<.0001 (17.848)**	1.311 (.373–4.606)	.673
≥3	84	82.9%	70.1%			
Clark's level						
I and II	47	100%	100%	**.002 (9.706)**	.667 (.216–2.063)	.482
III, IV and V	97	84.7%	71.4%			
Distant metastasis						
Present	34	67.5%	41.2%	**<.0001 (62.087)**	2.277 (.512–10.135)	.280
Absent	110	96.3%	92.8%			
AJCC-stage						
In situ	15	100%	100%	**<.0001 (71.219)**	1.438 (.650–3.181)	.369
I	51	100%	100%			
II	42	90.4%	80.1%			
III	23	69.3%	55.4%			
IV	13	69.2%	27.7%			
p-Akt1						
High expression	69	80.8%	64.2%	**<.0001 (24.206)**	2.766 (.952–8.038)	.062
Low expression	75	98.6%	94.1%			

CSS – Cancer–specific survival, HR – Hazard Ratio, AJCC – American Joint Committee on Cancer. Bold values indicate statistical significance ($P < .05$).

Cancer-specific survival (CSS) rates for cutaneous melanomas based on clinicopathological parameters are shown in Table 1. Significant prognostic factors for

CSS using a univariate model were Age (P = .013), Ulceration (P < .0001), Breslow's Thickness (P < .0001), Mitotic rate (P < .0001), Clark's level (P = .002), Distant metastasis (P < .0001), AJCC-stage (P < .0001) and p-Akt1 expression (P < .0001) Figure 1; while the unique independent prognostic factor in a multivariate analysis was Breslow Thickness (HR = 10.855, P = .041).

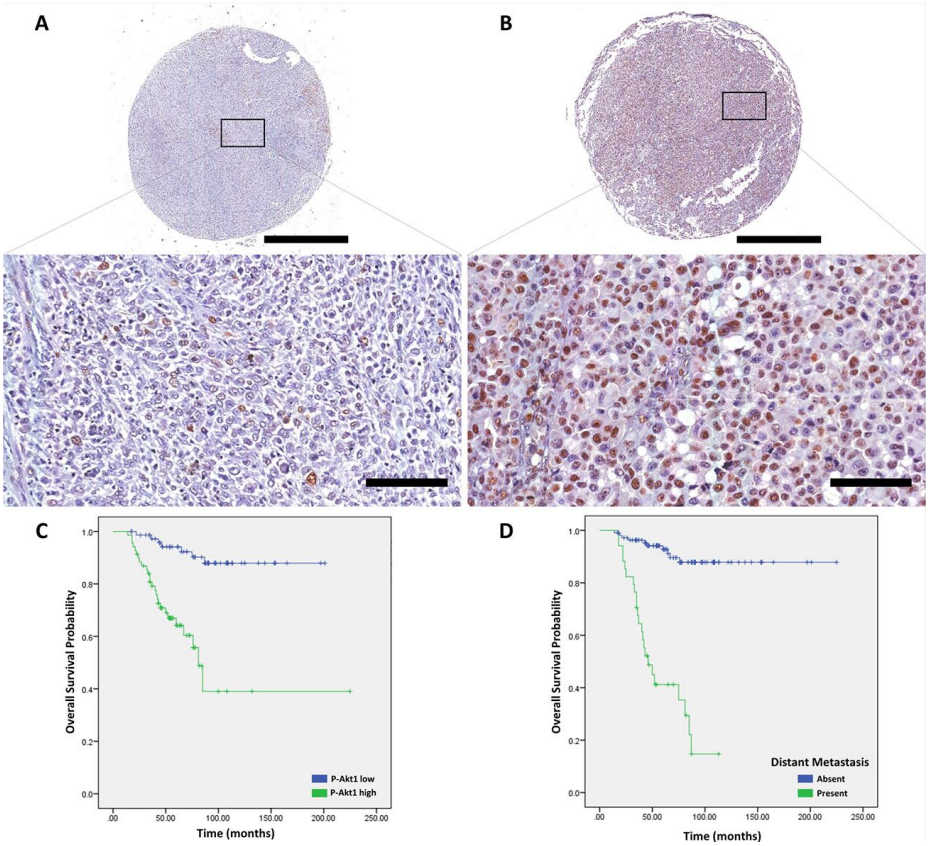

Figure 1: p-Akt1 expression in cutaneous melanomas. (A) Representative example of non-metastatic cutaneous melanoma in a tissue microarray, which was focally positive for p-Akt1. (B) Representative example of metastatic cutaneous melanoma in a tissue microarray, which was strongly positive for p-Akt1. (C) The association of p-Akt1nuclear expression and cancer-specific survival in cutaneous melanomas (logrank = 24.206, *P* < .0001). (D) The association of metastasis and cancer-specific survival in cutaneous melanomas (log-rank = 62.087, *P* < .0001). The scale bars represent 1 mm (top) and 100 μm (bottom).

p-Akt1 is an independent prognostic marker in Oral melanomas

For oral melanomas, 34 patients were enrolled in this study, with a mean age of 52 years, and regarding sex, 18 were men and 16 women. The main locations for oral melanomas were the hard palate (15/34), the tooth alveoluls (5/34), and other areas (14/34). The OM clinicopathological data are listed in Supplementary Table 2. The

median follow-up period was 23.5 months, ranging from 2 months to 14 years. The 3- and 5-year CSS rates were 49.1% and 35.8%, respectively.

Using the multivariate analysis, the mitotic rate (P = .001), presence of vascular invasion (P < .0001), neural invasion (P = .011), epithelioid cellular morphology (P = .001), and p-Akt1 nuclear expression were considered prognostic factors for CSS in oral melanomas (P < 0.001, Log-rank = 28.086; Figure 2). More interestingly, in the Cox regression model against established pathological prognostic factors such as mitotic rate, cellular morphology, vascular and neural invasion, p-Akt1 expression demonstrated to be an independent prognostic factor for oral melanomas Table 2 (HR = 11.397, P = .041).

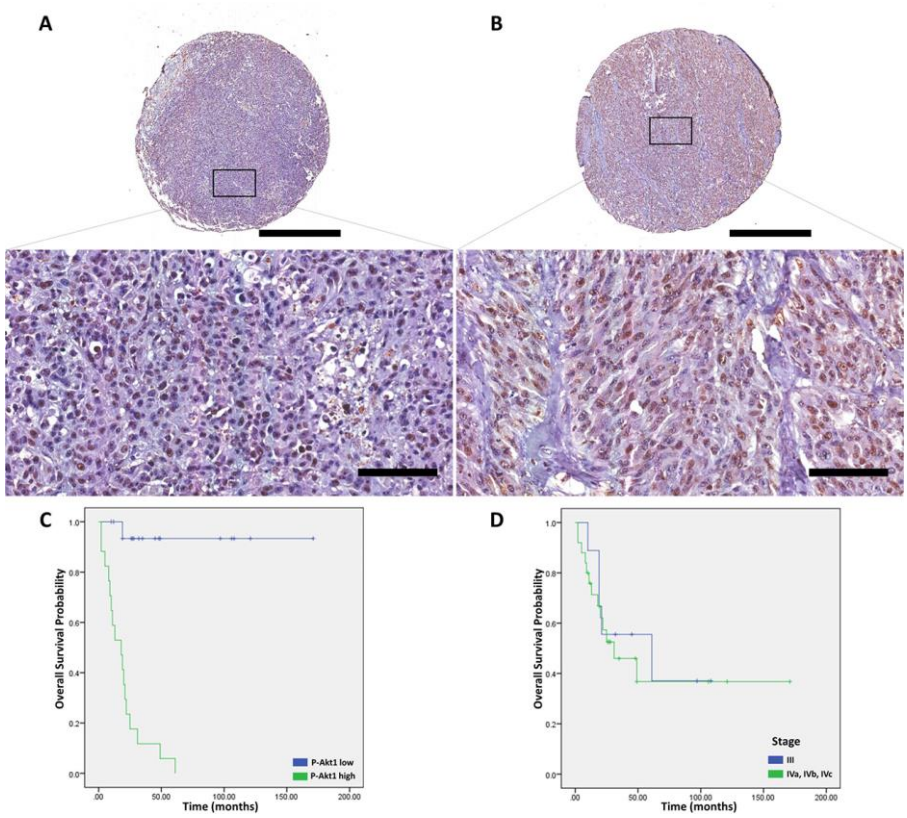

Figure 2: p-Akt1 expression in oral melanomas. (**A**) Representative example of oral melanoma in a tissue microarray, which was focally positive for p-Akt1. (**B**) Representative example of oral melanoma in a tissue microarray, which was strongly positive for p-Akt1. (**C**) The association of p-Akt1-nuclear expression and cancer-specific survival in oral melanomas (log-rank = 28.086, *P* < .0001). (**D**) The association of AJCC-stage and cancer-specific survival in oral melanomas (log-rank = .168, *P* = .682). The scale bars represent 1 mm (top) and 100 μm (bottom).

Table 2: The relationship between clinicopathological characteristics of patients with oral melanomas, p-Akt1 expression and cancer-specific survival

Factors	Sample (%)	CSS (%)		Univariate	Multivariate	
		3-year	5-year	*P* (log-rank)	HR (95% CI)	*P*-value
Age						
<47	18 (53.9)	53.8%	38.5%	.926 (.009)	1.407 (.237–8.361)	.707
≥47	16 (46.1)	41.5%	41.5%			
Gender						
Female	16 (46.1)	38.4%	38.4%	.374 (.791)	.718 (.196–2.628)	.617
Male	18 (53.9)	58.2%	38.8%			
Treatment						
Only surgery	22 (64.7)	55.4%	55.4%	.070 (3.273)	.689 (.129–3.674)	.663
Surgery + chemo/radiotherapy	11 (32.4)	38.1%	12.7%			
Anatomical site						
Palate	15 (44.1)	34.2%	34.2%	.113 (4.355)	–	–
Others	19 (55.9)	68.8%	55%			
Mitotic rate						
<1	14 (41.2)	85.1%	85.1%	**.001 (10.116)**	1.372 (.154–12.212)	.777
≥1	20 (58.8)	26.9%	13.5%			
Vascular invasion						

Factors	Sample (%)	CSS (%)		Univariate	Multivariate	
		3-year	5-year	*P* (log-rank)	HR (95% CI)	*P*-value
Absent	21 (61.8)	80.4%	58.6%	**<.0001 (18.408)**	1.485 (.191–11.579)	.706
Present	13 (38.2)	0%	0%			
Neural invasion						
Absent	26 (76.5)	42.9%	42.9%	**.011 (6.467)**	4.622 (.640–33.373)	.129
Present	8 (23.5)	15.6%	15.6%			
Cellular morphology						
Epithelioid	22 (64.7)	30.3%	20.2%	**.001 (12.052)**	.927 (.085–10.121)	.951
Non-epithelioid	12 (35.3)	90%	90%			
AJCC-stage						
III	9 (26.5)	55.6%	37%	.682 (.168)	.885 (.211–3.706)	.867
IVa, b and c	25(73.5)	46%	36.8%			
p-Akt1						
High expression	17 (50)	11.8%	5.9%	**<.0001 (28.086)**	**11.397 (1.102–117.855)**	**.041**
Low expression	17 (50)	93.3%	93.3%			

CSS – Cancer–specific survival, HR – Hazard Ratio, AJCC – American Joint Committee on Cancer. Bold values indicate statistical significance ($P < .05$)

p-Akt1 is an independent prognostic marker in sinonasal melanomas

Regarding the sinonasal melanomas (SNM), out of 31 patients, 15 were women and 16 men of ages between 24 and 82 (mean age, 55). The tumours were located at nasal cavity (41.9%), maxillary sinus (38.7%), and rhinopharynx (19.4%). Detailed clinicopathological data for SNM are shown in Supplementary Table 3. The median follow-up for SNM patients was 30 months, ranging from 3 months to 18.6 years, and the CSS 3-years and 5-years survival rates were 56.9% and 31.8%, respectively.

In a univariate model, vascular invasion (P = .042) and fusiform cellular morphology (P = .001) were associated with poorer CSS for patients with SNM (Table 3). p-Akt1 was associated with poor CSS for SNM in the univariate (P = .001, log-rank = 11.079) and multivariate (HR = 65.726, P < .0001) models Figure 3. In addition, the clinical grade was also an independent prognostic marker for SNM (HR = 7.351, P = .019, Table 3).

Table 3: The relationship between clinicopathological characteristics of patients with sinonasal melanomas, p-Akt1 expression and cancer-specific survival

Factors	Sample	CSS (%)		Univariate	Multivariate	
	(%)	3-year	5-year	*P* (log-rank)	HR (95% CI)	*P*-value
Age						
<58	16 (51.6)	60.6%	30.3%	.701 (.147)	1.935 (.479–7.820)	.354
≥58	15 (48.4)	51.3%	38.5%			
Gender						
Female	15 (48.4)	67%	41.9%	.319 (.994)	–	–
Male	16 (51.6)	49.2%	25.3%			
Treatment						
Only surgery	13 (41.9)	63.5%	25.4%	.604 (.268)	.473 (.132–1.694)	.250
Surgery + chemo/radiotherapy	17 (54.8)	52.7%	36.1%			
Anatomical site						
Nasal cavity	13 (41.9)	56.6%	45.3%	.239 (2.860)	–	–
Others	18 (58.1)	50%	0%			
Mitotic rate						
<1	20 (64.5)	60.2%	50.2%	.401 (.707)	.361 (.103–1.260)	.110
≥1	11 (35.5)	54.5%	10.9%			
Vascular invasion						
Absent	22 (70.9)	63.9%	45.7%	**.042 (4.151)**	.280 (.072–1.080)	.065
Present	9 (28.1)	44.4%	11.1%			
Neural invasion						
Absent	27 (87.1)	63.1%	38.5%	.072 (3.230)	3.223 (.616–16.857)	.166
Present	4 (12.9)	25%	0%			
Cellular morphology						

Factors	Sample	CSS (%)		Univariate	Multivariate	
	(%)	3-year	5-year	*P* (log-rank)	HR (95% CI)	*P*-value
Epithelioid	9 (29.1)	100%	62.5%	**.001 (17.600)**	.582 (.325–1.040)	.068
Fusiform	13 (41.9)	15.4%	7.7%			
AJCC-stage						
III	9 (28.1)	85.7%	64.3%	.060 (3.544)	**7.351 (1.392–38.821)**	**.019**
IVa, b and c	22 (70.9)	45%	20.2%			
p-Akt1						
High expression	20 (64.5)	42.2%	12.1%	**.001 (11.079)**	**65.726 (6.491–665.549)**	**<.0001**
Low expression	11 (35.5)	88.9%	88.9%			

CSS – Cancer–specific survival, HR – Hazard Ratio, AJCC – American Joint Committee on Cancer. Bold values indicate statistical significance ($P < .05$)

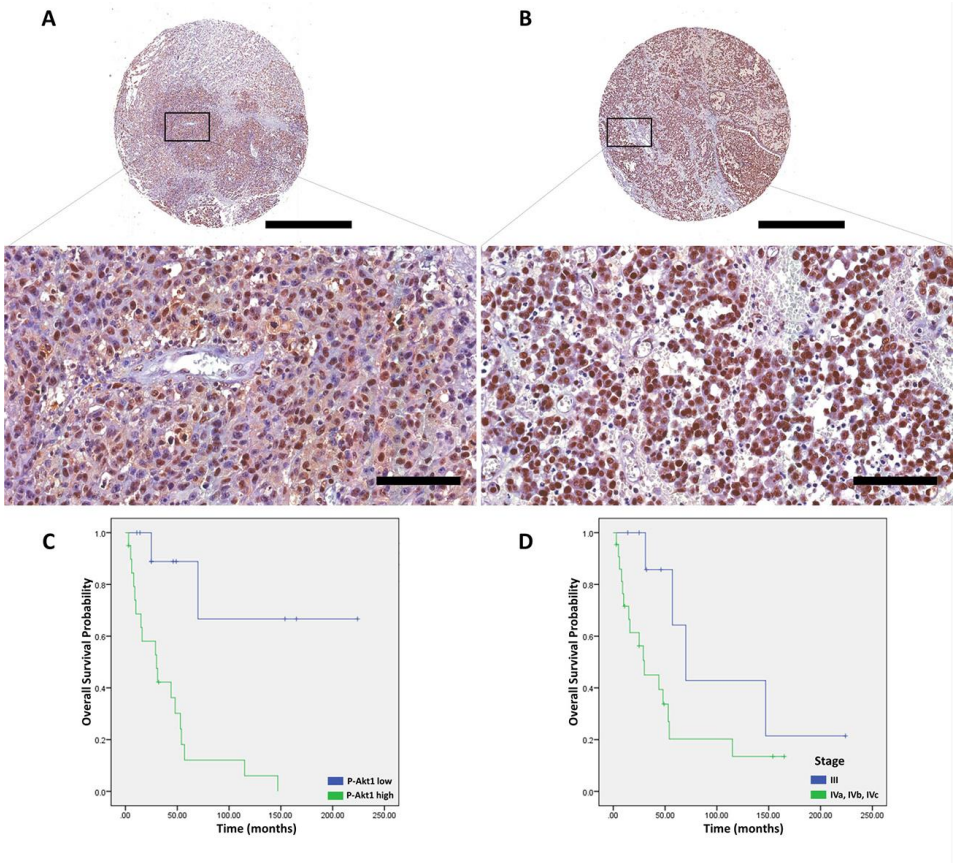

Figure 3: p-Akt1 expression in sinonasal melanomas. (A) Representative example of sinonasal melanoma in a tissue microarray, which was focally positive for p-Akt1. (B) Representative example of sinonasal melanoma in a tissue microarray, which was strongly positive for p-Akt1. (C) The association of p-Akt1-nuclear expression and

cancer-specific survival in sinonasal melanomas (log-rank = 11.079, P = .001). (D) The association of AJCC-stage and cancer-specific survival in sinonasal melanomas (logrank = 3.544, P = .060). The scale bars represent 1 mm (top) and 100 μm (bottom).

p-Akt1 expression is associated with clinicopathological parameters in melanomas

In the cutaneous melanomas patients, p-Akt1 showed positive association with ulceration (P < .0001), growth phase (P < .0001), Breslow's Thickness (P < .0001), mitotic rate (P = .003), Clark's Level (P < .0001), metastasis (P = .008), AJCC-stage (P < .0001), and recurrence (P < .0001), as shown in Table 4. For oral melanomas, p-Akt1 expression showed association with vascular invasion (P = .001), mitotic rate (P < .0001), and epithelioid cellular morphology (P < .0001), as displayed in Table 5. In addition, in the SNM cohort (Table 6), vascular invasion (P = .008), mitotic rate (P = .023), and fusiform cellular morphology (P = .009) were significantly associated with the positive p-Akt1-nuclear expression.

Table 4: The relationship between p-Akt1 nuclear expression and clinicopathological characteristics of 144 patients with cutaneous melanomas

Clinicopathological characteristics	**p-Akt1 low**	**p-Akt1 high**	***P*-value**
Age (<56/≥56)	44/31	34/35	.259
Gender (Female/Male)	39/36	32/37	.500
Site (Trunk/Head and neck/Upper limbs/Lower Limbs)	33/11/9/22	31/11/9/18	.975
Ulceration (Absent/Present)	63/12	20/49	**<.0001**
Growth phase (Radial/Vertical)	40/35	15/54	**<.0001**
Breslow's Thickness (<1.55/≥1.55)	59/16	13/56	**<.0001**
Mitotic rate (<3/≥3)	40/35	20/49	**.003**
Clark's Level (I and II/III, IV and V)	40/35	7/62	**<.0001**
Metastasis (Absent/Present)	64/11	46/23	**.008**
AJCC-stage (*in situ*/I/II/III/IV)	15/39/10/8/3	0/12/32/15/10	**<.0001**

Clinicopathological characteristics	**p-Akt1 low**	**p-Akt1 high**	***P*-value**
Recurrence (Absent/Present)	61/14	26/43	**<.0001**

Table 5: The relationship between p-Akt1 nuclear expression and clinicopathological characteristics of 34 patients with oral melanomas

Clinicopathological characteristics	**p-Akt1 low**	**p-Akt1 high**	***P*-value**
Age (<47/≥47)	8/9	10/7	.492
Gender (Female/Male)	8/9	8/9	1.000
Site (Palate/alveolus/others)	6/2/9	9/3/5	.379
Treatment (only surgery/surgery plus chemo-radiotherapy)	13/4	9/7	.151
Vascular invasion (Absent/Present)	15/2	6/11	**.001**
Neural invasion (Absent/Present)	14/3	12/5	.419
Mitotic rate (<1/≥1)	12/5	2/15	**<.0001**
Necrosis (Absent/Present)	14/3	10/7	.132
Cellular morphology (Non-epithelioid/Epithelioid)	12/5	0/17	**<.0001**
Recurrence (No/Yes)	14/3	13/4	.671

Table 6: The relationship between p-Akt1 nuclear expression and clinicopathological characteristics of 31 patients with sinonasal melanomas

Clinicopathological characteristics	**p-Akt1 low**	**p-Akt1 high**	***P*-value**
Age (<58/≥58)	5/6	11/9	.611
Gender (Female/Male)	7/4	8/12	.208
Site (Nasal Cavity/Maxillary sinus/Rhinopharynx)	6/5/0	7/7/6	.126

Clinicopathological characteristics	**p-Akt1 low**	**p-Akt1 high**	***P*-value**
Treatment (only surgery/surgery plus chemo-radiotherapy)	5/6	8/11	.741
Vascular invasion (Absent/Present)	11/0	11/9	**.008**
Neural invasion (Absent/Present)	11/0	16/4	.112
Mitotic rate (<1/≥1)	10/1	10/10	**.023**
Necrosis (Absent/Present)	8/3	10/10	.220
Cellular morphology (Epithelioid/Fusiform/Plasmacytoid-Undifferentiated)	6/2/3	3/11/6	**.009**
Recurrence (No/Yes)	7/4	12/8	.842

p-Akt1 expression is higher in metastatic cutaneous melanomas and mucosal melanomas than in non-metastatic cutaneous melanomas

p-Akt1 expression was tested in a large cohort of cutaneous, oral, and sinonasal melanomas, and it was expressed in tumour cells and occasionally in inflammatory cells surrounding the tumour. We also compared the scores of p-Akt1-positive nuclei into four groups: (a) primary (cutaneous melanoma) without metastatic history; and (b) primary (cutaneous melanoma) with metastatic history, (c) oral melanomas, and (d) sinonasal melanomas.

The p-Akt1 expression was predominantly detected in the nucleus, and non-metastatic cutaneous melanomas have a mean of 5.6% of the nuclei positive (ranging from 0% to 50.15%). Primary metastatic cutaneous melanomas have a mean of 15.7% (ranging from 1.7% to 41.3%). Cutaneous melanomas with low and high p-Akt1-nuclear expression are illustrated in Figure 1. The representative photomicrographs for p-Akt1 expression in oral and sinonasal melanomas are illustrated in Figures 2 and 3, respectively. Oral melanomas have scores of p-Akt1-positivity (mean = 19.45%, ranging from 2.6% to 44.4%) similar to the cutaneous melanomas with metastatic history. The sinonasal melanomas demonstrated a higher number of nuclei positive for p-Akt1 (mean = 56.15%, ranging from 15.1% to 82.6%), statistically different from the other subtypes of melanomas analysed in this cohort (Figure 4). The nuclear p-Akt1-

expression was closely related with invasive cells in all tumours, as illustrated in Figure 4.

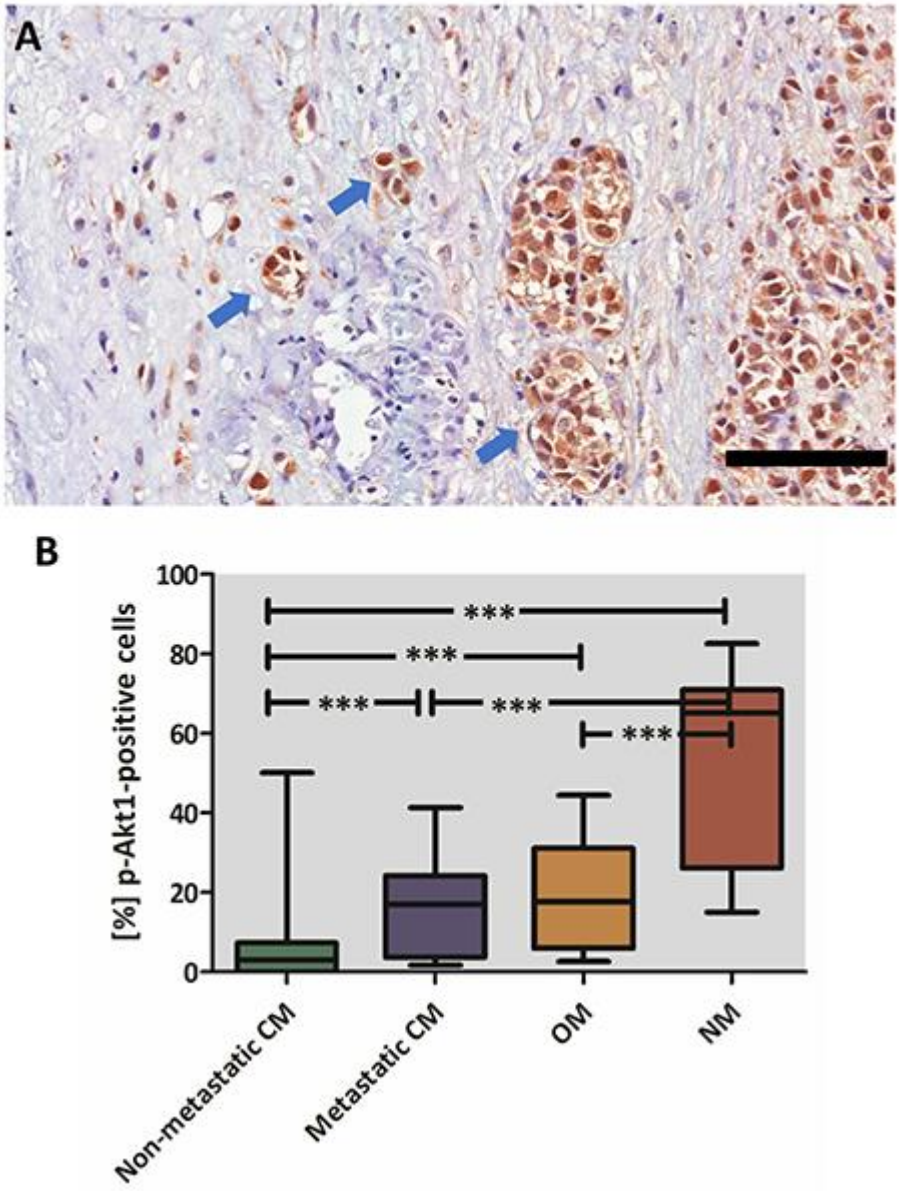

Figure 4: p-Akt1 expression in invasive cells and in different subgroups of melanomas. (**A**) Invasive cells strongly express p-Akt1 in the nucleus of a case of sinonasal melanoma. (**B**) Graphical representation of the median, minimum, and maximum scores of p-Akt1-nuclear positivity in the different subgroups of melanomas (***indicates statistically significant difference between the groups, ANOVA test, *P* value < .00001). The scale bar represents 100 μm.

Discussion

The poor prognosis of patients with mucosal melanomas and their ineffective treatment options evidence that these tumours have a distinct biological signature,

which is different from the other subtypes of melanomas [5, 10, 19]. Although some studies have extensively addressed the clinicopathological profile of the mucosal melanomas and their immunoprofile, these tumours have lacked predictive markers for tailored treatment [20–24]. Currently, the insufficient knowledge regarding molecular pathways that are selectively activated in different melanomas subtypes ensures comparative studies in mucosal and cutaneous melanomas. Herein, the authors proposed this comparative study to assess the p-Akt1 prognostic value in a series of cutaneous, oral, and sinonasal melanomas.

We used the AJCC system [25] for cutaneous melanomas staging. The AJCC-stage of cutaneous melanoma's patients significantly affects the outcome, indicating that it is a reproducible method for suggesting the prognosis and therapeutic options. In this study, we also used the AJCC 7th edition staging system for the mucosal melanomas. This system is more useful in prognosis prediction than the Ballantyne's staging system, which is mostly applied [19]. However, we used a dichotomized model (III/IV AJCC-stages), and the AJCC-staging had no impact on survival rates for oral melanoma patients, in the multivariate model. The relatively small number of cases, one limitation of our study, may explicate this finding. However, as mucosal melanomas of the head and neck are very rare tumours, the authors have provided novel information regarding sixty-five patients with oral and sinonasal melanomas.

Melanomas with predominant epithelioid morphology have been considered tumours of poor prognosis, as these neoplasms have greater DNA ploidy abnormalities when compared with other cellular morphologies in cutaneous melanomas [26]. Interestingly, in the current study, the epithelioid cellular morphology was found to be associated with a poor prognosis in oral melanomas, and it is in accordance with the findings from previous studies [27]. We also demonstrated a statistically significant association of the epithelioid cellular morphology with p-Akt1 overexpression, indicating a probable correlation between these two features. On the other hand, in sinonasal melanomas, only the undifferentiated cell morphology has been reported to confer poorer prognosis [28]. However, in this study, the spindle cell morphology was associated with worse prognosis for sinonasal melanoma patients.

The protein kinase B is serine/threonine-specific protein kinase involved in several biological activities in a wide range of cells, such as glucose metabolism and apoptosis [29]. To date, three isoforms of this protein have been well-described, AKT1, AKT2, and AKT3 [30]. AKT1 is widely documented for its activity in cellular

metabolism of several human cancers [29] and when phosphorylated, p-Akt1 is described as playing an important role in the redox modulation of cell cycle progression [31]. On the other hand, Akt2 seems to play a critical role in cell proliferation through increased glycogen synthesis [32] and Akt3 expression is reported in normal tissues, including the brain, heart, kidney, and fat [33].

Previous studies have demonstrated that p-Akt1-nuclear expression is closely associated with worse prognosis in breast [12], gastric [13], and oesophageal squamous carcinoma [14]. In opposition, it is associated with a favourable outcome in patients with pancreatic cancer [34]. The tumorigenic activity of p-Akt1 has also been investigated in several tumours [35–37] and in the context of tumour cells, Akt1 upregulates cell proliferation, invasion, and migration [38, 39]. Contrastively, its deletion prevents lung tumorigenesis in mice models [40]. One previous study shows the putative role of p-Akt1 in metastatic cutaneous melanomas [41]. In this study, the nuclear p-Akt1 expression was associated with the presence of distant metastasis in patients with cutaneous melanomas. In fact, several studies have addressed the role of p-Akt1 in cancer progression and metastasis [42], including its important part in the development of melanoma metastases in a mice model [18]. In addition, many studies have demonstrated the role of Akt1 in metastases development in several malignancies, such as colorectal [43] and lung [44] cancers. Although a correlation between p-Akt1 expression and the metastatic status in cutaneous melanomas have been found for these tumours, the p-Akt1 showed prognostic values only in the univariate model, but it was lost in the multivariate model. In addition, p-Akt1 expression was correlated with important clinicopathologic features in the cutaneous melanomas, such as Breslow's thickness, Clark's level, mitotic rate, and AJCC-stage, indicating that p-Akt1 is correlated with melanoma tumorigenesis and affects the clinical outcome of these patients, emerging as a possible target for personalized therapies.

For oral and sinonasal melanomas, univariate and multivariate analysis identified p-Akt1 as an independent prognostic factor. Our results also showed that p-Akt1 expression was significantly higher in sinonasal melanomas than in oral and cutaneous melanomas. Taken together, these data support the hypothesis that sinonasal melanomas have distinct molecular signature and biological behaviour from its cutaneous counterpart. Importantly, we have demonstrated by immunohistochemistry that p-Akt1 is overexpressed in the nuclei of tumour cells and is closely correlated with poor outcome in a subset of mucosal melanomas, whereas the cutaneous melanomas

demonstrated lower p-Akt1 expression compared with the oral and sinonasal melanomas. In fact, several studies have addressed the differences concerning etiologic factors and molecular pathways involved in the pathogenesis of cutaneous vs mucosal melanomas [3–5, 45]. For example, c-KIT aberrations were reported in tumours that have no correlation with chronic sun exposure (mainly mucosal melanomas). In opposition, the cutaneous counterpart frequently harbours mutations in the BRAF gene, given the fact that chronic sun exposure is important for its pathogenesis [9, 46]. It has been previously demonstrated that c-KIT is a key molecule for p-Akt1 activation through PI3K phosphorylation [46]. Therefore, particularly in mucosal melanomas, a correlation between p-Akt1 overexpression and c-KIT mutation may help to understand how these pathways are important for melanoma pathogenesis and patient outcomes.

In the cohort of sinonasal melanomas analysed in this study, p-Akt1 overexpression showed a correlation with higher number of mitosis, presence of vascular invasion, and with spindle and undifferentiated cellular morphology. These findings provide new insights about the probable role of p-Akt1 in the aggressive genotype and phenotype of the sinonasal melanomas. In these particular tumours, necrosis was associated with overall survival in one previous report [47]. However, in this study's cohort, necrosis has not demonstrated prognostic predicting value, corroborating with other previous studies [48, 49]. No correlation between p-Akt1 and necrosis was observed in this study.

Overall, the treatment of choice for both mucosal and cutaneous melanoma is the wide surgical resection [50–52]. Although it has been demonstrated that melanomas are not radiosensitive, some tumours have been treated with adjuvant radiotherapy, and the use of these combined therapies are not standardized [10]. Regarding the mucosal melanomas, approximately 30% of the patients from the sample with oral melanomas and 50% of the patients with sinonasal melanomas received chemotherapy or radiotherapy as adjuvant treatment. Nevertheless, no differences in the cancer-specific survival were observed in both groups. The use of adjuvant therapies with surgical resection still needs to be investigated as a option to treat patients with mucosal melanomas [53].

In conclusion, the results of the current study demonstrated that p-Akt1 overexpression is an independent prognostic marker in mucosal melanomas and is significantly up-regulated in sinonasal melanomas. As both mucosal and metastatic cutaneous melanomas showed high frequency of p-Akt1 expression, our findings

suggest that mucosal melanomas have a biological behaviour that is similar to the one identified in aggressive cutaneous melanomas.

Abbreviations

CM: cutaneous melanomas; OM: oral melanomas; SNM: sinonasal melanomas; HR: Hazard Ratio; AJCC: American Joint Committee on Cancer; p-Akt1: Phosphorylated Akt1, or protein B kinase 1; IHC: immunohistochemistry; CSS: Cancer-specific survival; DFS: disease-free survival.

Author contributions

Conception and design: CDS, JJ, OPA. Provision of study materials and/or patients: RC, FVMBC, MBC, RRDR, LSA, AMMA, MGFC, OPA. Collection and assembly of data: CDS, TMLM, MGFC, OPA, JJ. Data analysis and interpretation: CDS, TMLM, FVMBC, AMMA, JJ, Manuscript writing: CDS, TMLM. Final approval of manuscript: All authors approved the definitive version of this manuscript.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to disclose.

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Supplementary Table 1. Clinicopathologic features of 144 patients with cutaneous melanomas.

Factors	Category	Frequency *n (%)*
Sex	Female	71 (49.3)
	Male	73 (50.7)
Age (years)	Range; median	20–88; 56
	Mean (SD)	56 (15.7)
Site	Head and neck	22 (15.3)
	Trunk	64 (44.4)
	Upper limbs	18 (12.5)
	Lower limbs	40 (27.8)
T-stage	In situ	15 (10.4)
	I	51 (35.4)
	II	42 (29.2)
	III	23 (16.0)
	IV	13 (9.0)
Clark's level	I and III	47 (32.6)
	III, IV and V	97 (67.4)
Ulceration	Present	61 (42.4)
	Absent	83 (57.6)
Mitotic index (number/mm²)	<3	60 (41.7)
	≥3	84 (58.3)
Breslow's thickness (mm)	<1.55	72 (50)
	≥1.55	72 (50)
Distant metastasis	Present	34 (23.6)
	Absent	11 (76.4)

Supplementary Table 2. Clinicopathologic features of 34 patients with oral melanomas.

Factors	Category	Frequency *n (%)*
Sex	Female	16 (47.1)
	Male	18 (52.9)
Age (years)	Range; median	19–89; 47
	Mean (SD)	52 (19.5)
Site	Palate	15 (44.1)
	Inferior gingiva	5 (14.7)
	Other	14 (41.2)
Cell morphology	Epithelioid	22 (64.7)
	Non-Epithelioid	12 (35.3)
Vascular invasion	Present	13 (38.2)
	Absent	21 (61.8)
Neural invasion	Present	8 (23.5)
	Absent	26 (76.5)
Necrosis	Present	10 (29.4)
	Absent	24 (70.6)
Mitotic index	<1	14 (41.2)
(number/mm²)	≥1	20 (58.8)
Treatment	Only surgery	22 (64.7)
	Surgery plus chemotherapy or radiotherapy	11 (32.4)
	Other/missing	1 (2.9)
Clinical Stage	III	9 (26.5)
	IVa	16 (47.1)
	IVb	4 (11.8)
	IVc	5 (14.7)

Supplementary Table 3. Clinicopathologic features of 31 patients with sinonasal melanomas.

Factors	Category	Frequency *n (%)*
Sex	Female	15 (48.4)
	Male	16 (51.6)
Age (years)	Range; median	24–82; 58
	Mean (SD)	55 (17)
Site	Nasal cavity	13 (41.9)
	Maxillary sinus	12 (38.7)
	Rhinopharynx	6 (19.4)
Cell morphology	Epithelioid	9 (29.0)
	Fusiform	13 (41.9)
	Plasmacytoid	4 (12.9)
	Undifferentiated	5 (16.1)
Vascular invasion	Present	9 (28.1)
	Absent	22 (71.9)
Neural invasion	Present	4 (12.9)
	Absent	27 (87.1)
Necrosis	Present	13 (41.9)
	Absent	18 (58.1)
Mitotic index	<1	20 (64.5)
(number/mm²)	≥1	11 (35.5)
Treatment	Only surgery	13 (41.9)
	Surgery plus chemotherapy or radiotherapy	17 (54.8)
	Other/missing	1 (3.3)
Clinical stage	III	9 (29.0)
	IVa	11 (35.5)
	IVb	4 (12.9)
	IVc	7 (22.6)

2.2 Artigo: Cyclooxygenase 2 is associated with aggressiveness and unfavorable survival in oral melanoma

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Running tittle: COX-2 expression in oral melanoma.

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ABSTRACT

Objectives: The aim of this study was to investigate the association of cyclooxygenase 2 (COX-2) expression with clinicopathological data and survival. *Materials and methods:* immunohistochemical (IHC) staining was used to measure the expression of COX-2 on the tissue microarray from 30 patients with oral melanoma. A digital analysis was performed by slides scanning and scores of staining were assessed. The immunostaining scores were correlated with clinical parameters. Survival data were evaluated by log-rank test and COX proportional hazards regression analysis. *Results:* All oral melanomas were positive for COX-2 in different intensities. COX-2 expression was significantly associated with overall survival in a multivariate model [HR = 6.6475 (2.0466-21.5919); P-value = 0.0016]. *Conclusions:* Our results suggest that COX-2 may be considered a valuable prognostic marker for oral melanoma. These findings may help the development of target-based therapies focusing on COX-2 inhibitors as effective single or combined therapy against oral melanoma.

Keywords: Melanoma; oral; COX-2; prognosis.

INTRODUCTION

Cyclooxygenases (COXs) are enzymes responsible by converting the first step of arachidonic acid to prostaglandins and constitutes two subtypes, wherein COX-1 is mostly observed in normal tissue due to its association with physiological processes, while COX-2 is not detected in normal tissues, but is immediately induced by inflammatory reactions [1,2]. COX-2 is also expressed in several tumour types and its level of expression are associated with proliferation in non-small cell lung cancer [3], angiogenesis in pancreatic ductal adenocarcinoma [4], cell migration and invasion in hepatocellular carcinoma [5] and promotes apoptotic resistance in colorectal cancer [6]. COX-2 expression has already been demonstrated in oral and cutaneous metastatic melanoma [1,2,8], but the clinical significance of COX-2 expression in oral melanomas was not addressed in these articles.

Therefore, in this current study we sought to elucidate the prognostic significance of COX-2 overexpression in oral melanomas.

METHODS

This protocol of this study followed the ethical principles stated in the Declaration of Helsinki and was approved by the National Commission for Ethics in Research (CONEP-Brazil, CAAE: 72077517.1.0000.5418). We have included 30 patients with oral melanoma from four pathology laboratories which presented complete clinical information and follow-up. The lesions were diagnosed following the 4th edition for Mucosal Melanoma of the World Health Organization Classification of Head and Neck Tumours criteria [9]. In amelanotic or doubt cases, S-100, MelanA, and HMB45 immunostainings were performed. The staging classification was performed according to the 8th edition of the American Joint Committee on Cancer for Melanoma Staging [10].

For the immunohistochemical (IHC) polymer-based method accordingly protocols stablished in our laboratory [8,11]. We used the primary antibody COX-2 (Clone: CX-294; Dako Corporation, Carpinteria, CA, USA). Following the IHC reactions, the slides were scanned into high-resolution images and digitally assessed as described previously [8,11]. The immunostaining scores were correlated with clinical data using contingency tables and Chi- Square or Fisher's exact test. Survival curves were calculated according to the Kaplan-Meier method. The Log-Rank test was applied for patients with low and high markers expression (cut off value: median of positivity scores). COX proportional hazards regression analysis was performed to test the statistical independence and significance between pathological, molecular, and clinical variables. All statistical tests were carried out in the SPSS software, version 22.0 (SPSS Inc., Chicago, IL, USA) with a 95%-confidence level (P-value ≤ 0.05).

RESULTS

The current samples have already been used in other previous studies [8,11]. The mean age was 54 years old (range of 22-89 years-old); females were slight more affected than males (M:F ratio of 1:1.14). Regarding the anatomical site, palate was more affected (13 cases; 43.4%), followed by gingiva (5 cases; 16.6%). In addition, patients were mainly treated with only surgery (20 cases; 66.6%) and surgery plus chemotherapy or radiotherapy (9 cases; 30%). Regarding the tumour's AJCC-stage classification, the cases were stage III in 43.4%, IVa in 36.6% and IVb/IVc in 20%.

COX-2-positive cells showed reddish granules in the cytoplasm, evidenced by a variable cytoplasmic staining in all analysed samples. Its high expression was

significantly associated with vascular invasion (P-value: 0.0018), high mitotic index (P-value: 0.0344), epithelioid cellular morphology (P-value: 0.0032) and advanced AJCC-stage level (P-value: 0.0043). The univariate and multivariate Cox model revealed that none of the analysed factors was significantly associated with DFS. Concerning OS, the univariate model evaluation revealed that anatomical site (P-value: 0.0239), treatment (P-value: 0.0004), vascular invasion (P-value: < 0.0001), neural invasion (P-value: 0.0341), mitotic index (P-value: 0.0002), epithelioid cellular morphology (P-value: 0.0018), advanced AJCC-stage (P-value: 0.0007) and high COX-2 expression (P-value: 0.0003) showed significant results. The multivariate Cox model revealed that only high COX-2 expression was significantly associated with overall survival (P-value: 0.0016) (Table 1).

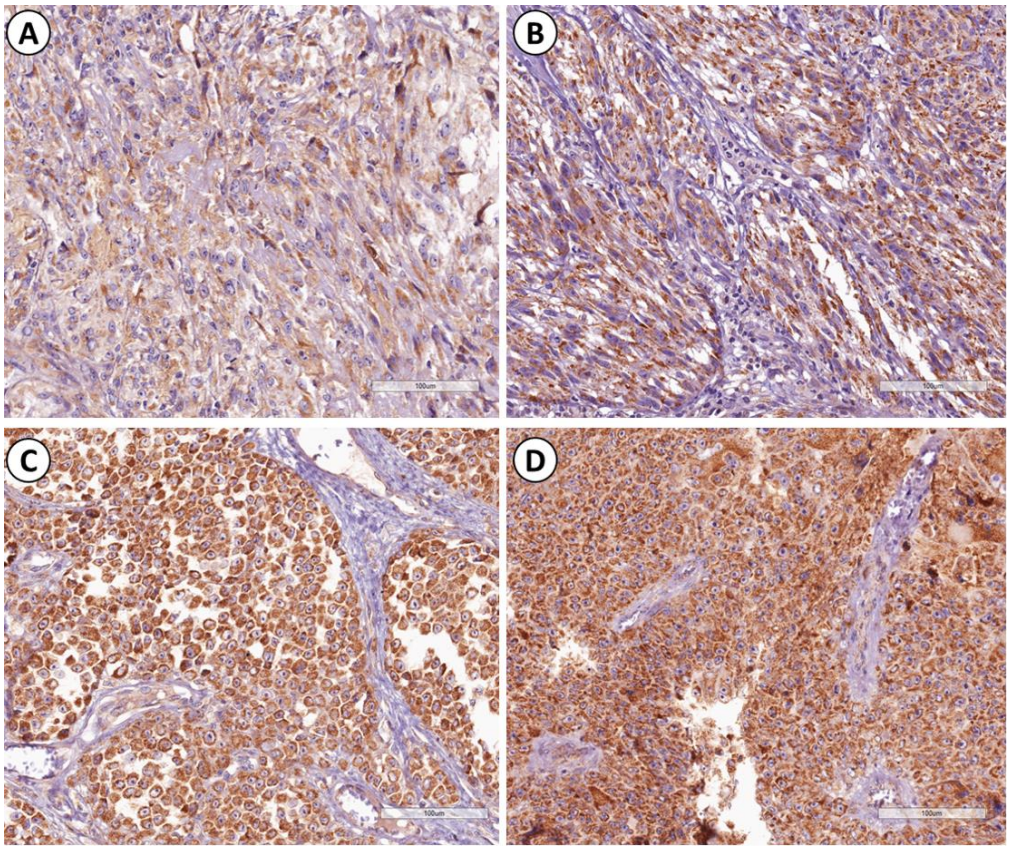

Figure 1. Immunohistochemical expression of COX-2 in oral melanomas. A-B: Positive expression in the cytoplasm of tumour cells of a predominantly spindle cell oral melanoma – a (weak), b (moderate). C-D: COX-2 demonstrated intense positivity in all epithelioid oral melanomas.

Table 1. Clinicopathological characteristics and survival analysis of all patients with oral melanoma.

	Categories		DFS univariate (%)			Multivariate		OS univariate (%)			Multivariate	
Variables		n (%)	3-years	5-years	P-value (logrank)	HR (95% CI)	P-value	3-years	5-years	P-value (logrank)	HR (95% CI)	P-value
Age	<54	16 (53.3)	88.8	71.6	0.0873	4.0731 (2.4287-6.8313)	0.8309	53.3	40.1	0.7634	1.0568 (0.0867-12.8837)	0.9654
	≥54	14 (46.7)	63.5	35.8				43.8	-			
Gender	Female	16 (53.3)	78.7	78.7	0.7574	127.4771 (7.2946-228.1245)	0.9415	38.3	38.3	0.3318	1.7371 (0.2743-11.0008)	0.5575
	Male	14 (46.7)	82	46.8				64.3	42.8			
Anatomical site	Palate	13 (43.4)	63	63	0.1808	0.0011 (2.0369-9.6351)	0.8407	27.7	27.7	0.0239*	0.3580 (0.0986-1.2998)	0.1184
	Gingiva	5 (16.6)	50	50				40	40			
	Others	12 (40)	100	66.6				81.8	65.4			
Treatment	Only surgery	20 (66.6)	86.2	71.9	0.3679	484.1991 (4.3047-5.4464)	0.8654	57.4	57.4	0.0004*	1.9265 (0.5707-6.5024)	0.2907
	Surgery plus CH or RT	9 (30)	64.3	42.8				44.4	14.8			
	Others	1 (0.4)	-	-				-	-			
Vascular invasion	Absent	19 (63.3)	87.4	56.6	0.1161	5.8936 (2.8031-1.2392)	0.8709	83.6	60.9	< 0.0001*	1.0944 (0.0943-12.6992)	0.9425
	Present	11 (36.6)	-	-				-	-			
Neural invasion	Absent	24 (80)	82.2	53.3	0.4965	1596.5338 (6.5659-3.8821)	0.9562	60.4	52.8	0.0341*	1.4340 (0.2214-9.2879)	0.7052
	Present	6 (20)	-	-				-	-			
Mitotic index	<1	13 (43.4)	90	72	0.1558	23.7592 (4.9228-	0.9613	91.6	91.6	0.0002*	10.9201 (0.6079-	0.1047
	≥1	17 (56.6)	72.2	36				23.5	17.6			

						1.1467)					196.1630)	
Necrosis	Absent	22 (73.3)	74.2	49.9	0.4002	0.0002 (1.0136-8.2176)	0.8731	51.2	41	0.5963	0.6026 (0.0337-10.7745)	0.7307
	Present	8 (26.6)	100	100				50	50			
Cellular morphology	Non-epithelioid	10 (33.3)	76.2	76.2	0.1756	0.000001 (1.5589-6.6615)	0.8468	88.8	88.8	0.0018*	2.4372 (0.2008-29.5684)	0.4841
	Epithelioid	20 (66.6)	84.8	42.4				35	23.3			
AJCC-stage	III	13 (43.4)	80.2	50.1	0.4554	6384.8337 (9.8415-4.1423)	0.7957	68.3	68.3	0.0007*	2.5116 (0.7057-8.9386)	0.1550
	IVa	11 (36.6)	88.8	59.2				60.6	40.4			
	IVb/IVc	6 (20)	-	-				-	-			
COX-2	Low	19 (63.3)	86.5	67.3	0.1875	3.7241 (0.4669-29.6992)	0.2145	78.3	68.5	0.0003*	6.6475 (2.0466-21.5919)	0.0016*
	High	11 (36.6)	-	-				-	-			

*Statistically significant difference
DFS – disease-free survival; OS – overall survival

DISCUSSION

Despite the better understanding of the mechanisms responsible for melanoma progression in recent years, the five-year survival rate of melanoma remains less than 25% throughout the world [1,6,10]. Particularly, mucosal melanomas represent a highly aggressive subset of all melanomas and due their rarity, in the last decades, it has been observed slightly advance on understanding the molecular pathways of interest in these tumours [12-14]. In the present study, we evaluated the expression of COX-2 in oral melanomas and its association with clinicopathological factors and survival rate.

A higher expression of COX-2 was significantly related with presence of vascular, high mitotic index and neural invasion. Previous studies have demonstrated a positive correlation between COX-2 expression with cellular proliferation and angiogenesis, since inflammation provides a conducive environment for tumour progression [3,4]. These factors are known to determine tumour progression and these results are consistent with previous literature which showed that COX-2 overexpression is associated with advanced disease [15].

The Cox multivariate model demonstrated that COX-2 expression was an independent prognostic factor for overall survival, evidencing the most significant result of our study. Previously, de Souza do Nascimento and collaborators showed COX-2 is a useful marker to differentiate oral nevi and oral melanoma [2]. In addition, Panza *et al.* [1] observed that high COX-2 expression is a negative prognostic factor in lymph node metastatic melanoma. To the best of our knowledge, this is the first study to show that COX-2 expression is associated with OS in oral melanoma. Our findings propose a useful tool to argue for a possible therapeutic use of prophylactic anti-inflammatory use in the treatment of oral melanoma [16-19]. It has been recognized a synergic effect of COX-2 inhibitors and chemotherapy and/or radiotherapy in some molecular targets, such as tumour growth and proliferation, including VEGFR or aromatase inhibitors [20-23].

In conclusion, we suggest COX-2 as valuable immunohistochemical prognostic marker for oral melanoma. Future studies addressing the molecular signals that regulate COX-2 in oral melanoma are necessary to evaluate the effect of novel therapeutic agents in the modulation of cell immune responses.

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2.3. Artigo: Oral amelanotic melanomas: clinicopathologic features of 8 cases and review of the literature

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Running tittle: A series of amelanotic oral melanomas.

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ABSTRACT

Background: Mucosal melanomas are very aggressive tumors, rarely observed in the oral cavity. Usually, it is identified based on the clinical and microscopical features, including variable amounts of melanin-pigmentation. However, when melanin is absent, the tumors are denominated amelanotic, presenting a tendency to misdiagnosis and delayed treatment.

Objective: To describe the clinicopathological characteristics of a series of oral amelanotic melanomas (OAM).

Methods: Cases diagnosed as OAM were retrospectively retrieved from the files of four south and central America institutions from January 2002 to January 2019. Data on clinical features, morphological aspects, immunohistochemical reactions, treatment and follow-up status were also collected from the patient's medical records.

Results: The study group included six male and two females (ratio of 3:1) ranging in age from 33 to 77 years (mean 53.6 years). Clinically, masses or ulcerated swellings were reported. The most common intraoral locations of the tumors were gingiva and palate. Cervical lymph node metastasis was observed in three patients at the time of diagnosis. Three patients were treated with surgery plus chemotherapy, two with only surgery and one patient received adjuvant radiotherapy. All but one patient died from complications of the tumors after a mean follow-up period of 8.5 months. (ranging from 2 to 23 months).

Conclusion: OAM is a very aggressive malignant tumor, it demands especial concern to early diagnosis which may achieve successful treatment, improving the patient's prognosis. Similarly to occurs in the cutaneous melanomas, the oral amelanotic counterpart seems to have a different biology from the pigmented ones.

INTRODUCTION

Oral melanomas are extremely rare malignant melanocytic neoplasms, representing about 2–8% of all melanomas [1,2]. Although the microscopic recognition of melanomas is usually possible by the combination of cytomorphological and architectural features, its diagnosis remains a challenge, especially in amelanotic or hypopigmented cases [3]. Amelanotic melanomas are exceedingly rare and can, clinically and microscopically, resemble other cancer types or even mimic benign tumors [1,4].

Recent evidences have demonstrated that cutaneous amelanotic melanomas have poorer prognosis than pigmented tumors [5]. However, few retrospective cohort studies have been carried out to evaluate the clinicopathological features of oral amelanotic melanomas (OAM). The aim of the present study was to describe the clinical, histological and immunoprofile features of a series of 8 OAM.

PATIENTS AND METHODS

Cases diagnosed as oral amelanotic melanomas were retrieved from the files of 2 Brazilian, 1 Mexican and 1 Guatemalan oral pathology services. Clinical data were retrieved from the pathology reports and follow-up information was obtained from the referring physicians. Five-micrometer hematoxylin and eosin–stained sections were reviewed for histological description of the lesions. Immunohistochemical reactions were performed in 3-μm tissue sections in silanized slides, using standard protocols for the following antibodies: S-100 (polyclonal, dilution 1:10.000), HMB-45 (clone HMB-45, dilution 1:200), Melan A (clone A103, dilution 1:800), pan-Cytokeratin (clone AE1/AE3, dilution 1:400), Vimentin (clone Vim 3B4, dilution 1:400), α-SMA (clone 1A4, dilution 1:400), CD45 LCA (clone 2B11+PD7/26, dilution 1:200), and Ki-67 (clone MIB-1, dilution 1:100). All antibodies were obtained from Dako (Glostrup, Denmark).

This study was carried out in accordance with the Helsinki Declaration of 1964 and was approved by the Piracicaba Dental School ethics board (#72077517.1.0000.5418).

RESULTS

Clinical features

The patients included 6 men and 2 women, with a mean age at presentation of 53.6 years (range 33–77 years). The patients were white (n = 2), black (n = 5) or unknown (n

= 1) race. The locations of the tumors were upper and lower gingiva (n=3), tongue (n=1), lip (n=1), upper alveolar ridge (n=1), and palate (n=2). The tumors presented as reddish swellings or ulcerations and lacked brown or dark pigmentation in oral cavity. One patient complained of bleeding from the lesion, and three patients (37.5%) had lymph node involvement or metastasis at the time of diagnosis (Table 1).

Table 1. Summary of clinical features of 8 cases of oral amelanotic melanomas.

Case	Age (years)	Gender	Location	Treatment	Follow-up	Survival time (months)	Lymph node involvement
1	34	M	Soft palate	SX+CX+RX	died	2	Yes
2	77	M	Tongue	SX+CX	died	11	No
3	56	M	Lip	SX	disease-free	23	No
4	55	M	Inferior Gingiva	SX	died	5	No
5	52	F	Superior Gingiva	SX+CX	died	8	Yes
6	33	F	Inferior Gingiva	SX+CX	died	2	No
7	68	M	Superior alveolar ridge	N/A	lost	N/A	N/A
8	54	M	Palate	N/A	lost	N/A	Yes

M – male, F – Female, SX – surgery, CX – chemotherapy, RX – radiotherapy, N/A – Not available.

Clinically, case 1 presented as a diffuse and painless asymptomatic swelling (approximately 3.0 cm of size) covered by erythematous lining mucosa. The tumor extended to the oropharynx region and caused difficulty in swallowing and phonation. In addition, extraoral clinical examination showed ipsilateral nodal metastasis at the time of diagnosis. Case 6 presented as a small asymptomatic sessile nodule, 0.5 cm, bleeding on palpation, in the region of the incisive papilla between lower central incisors with a clinical diagnosis of pyogenic granuloma. The case 7 presented as an asymptomatic swelling showing areas of ulceration in the upper left alveolar ridge. A panoramic radiograph showed slight erosion and alteration of the trabecular bone of the affected region. The patient reported being a smoker and a chronic alcoholic (Fig. 1).

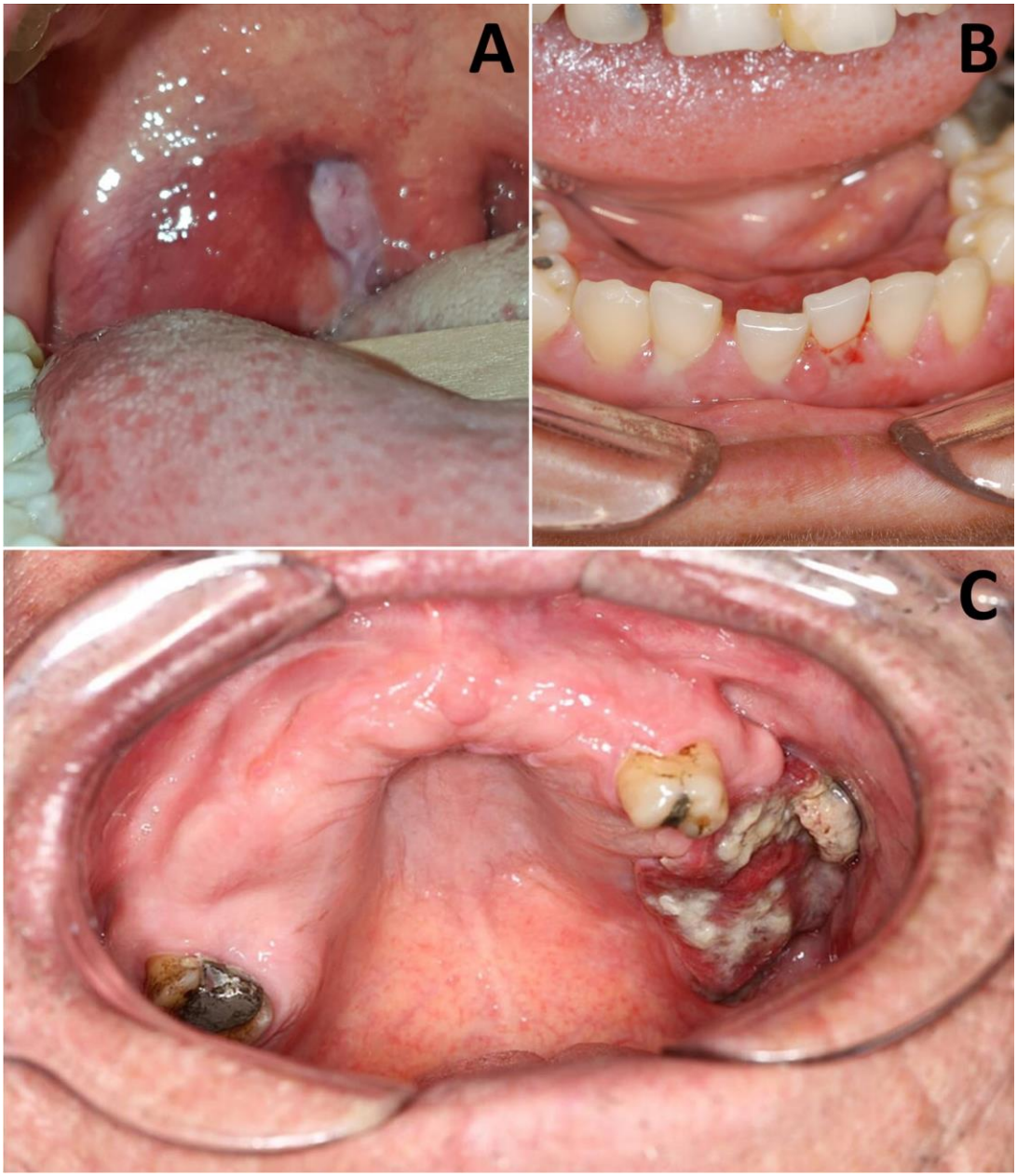

Figure 1. A: Clinical presentation of the case 1, a diffuse and painless asymptomatic swelling (approximately 3.0 cm of size) covered by erythematous lining mucosa. The tumor extended to the oropharynx region and caused difficulty in swallowing and phonation. B: In this case 6, the patient presented a small asymptomatic sessile nodule, 0.5 cm, bleeding on palpation, in the region of the incisive papilla between lower central incisors with a clinical diagnosis of pyogenic granuloma. C: In the case 7, the patient presented an asymptomatic swelling showing areas of ulceration in the upper left alveolar ridge.

Differential clinical diagnoses for all lesions included: infectious diseases, malignant neoplasms such as squamous cell carcinoma, lymphomas and others malignant tumors. For the incisive papilla lesion, periodontal disease and pyogenic granuloma were raised. In none of the 8 cases the dentist specifically mentioned melanoma as part of the clinical differential diagnosis. Some cases that had the

diagnosis of benign lesions were completely excised; whereas cases with clinical appearance of malignant lesions were submitted to incisional biopsy.

Pathologic features

Histologically all cases demonstrated a diffuse proliferation of pleomorphic rhabdoid, spindle and epithelioid melanocytes with a variable number of mitosis and absent or very focal melanin deposition (Figs. 2 and 3). In two cases, undifferentiated small blue round cells were observed. In addition, nuclear inclusions and stromal desmoplasia areas were also observed in one case. Large central amphophilic or eosinophilic nucleoli were observed in 5 cases. Lymphomas, carcinomas, and sarcomas not otherwise specified were considered as the differential diagnosis. Regarding cell morphology, 3 (37.5%) presented a predominance of undifferentiated cells, 3 (37.5%) epithelial/rhabdoid and 2 (25.0%) with predominant spindle cell morphology (Table 2). Morphological features of the cases 5 and 7 are illustrated in the Figure 2 and 3, respectively.

Table 2. Microscopical and immunohistochemical features of 8 oral amelanotic melanomas.

Case	Predominant morphology	Vim	AE1/AE3	LCA	α-SMA	S-100	Melan A	HMB-45	Ki-67
1	Undifferentiated	+	neg	neg	neg	+	weak/neg	+	>70%
2	Epithelioid	+	neg	neg	neg	+	+	+	>70%
3	Spindle cell	+	neg	neg	neg	+	+	+	22.1%
4	Undifferentiated	+	neg	neg	neg	+	weak/neg	+	>70%
5	Epithelioid	+	neg	neg	neg	+	+	+	34.2%
6	Spindle cell	+	neg	neg	neg	+	+	+	>90%
7	Undifferentiated	+	neg	neg	neg	+	+ strong	+	>70%
8	Epithelioid	+	neg	neg	neg	+	+	+	51.4%

Vim – vimentin, AE1/AE3 – pan-cytokeratin, LCA – leucocyte common antigen, α-SMA α-smooth muscle actin, neg – negative

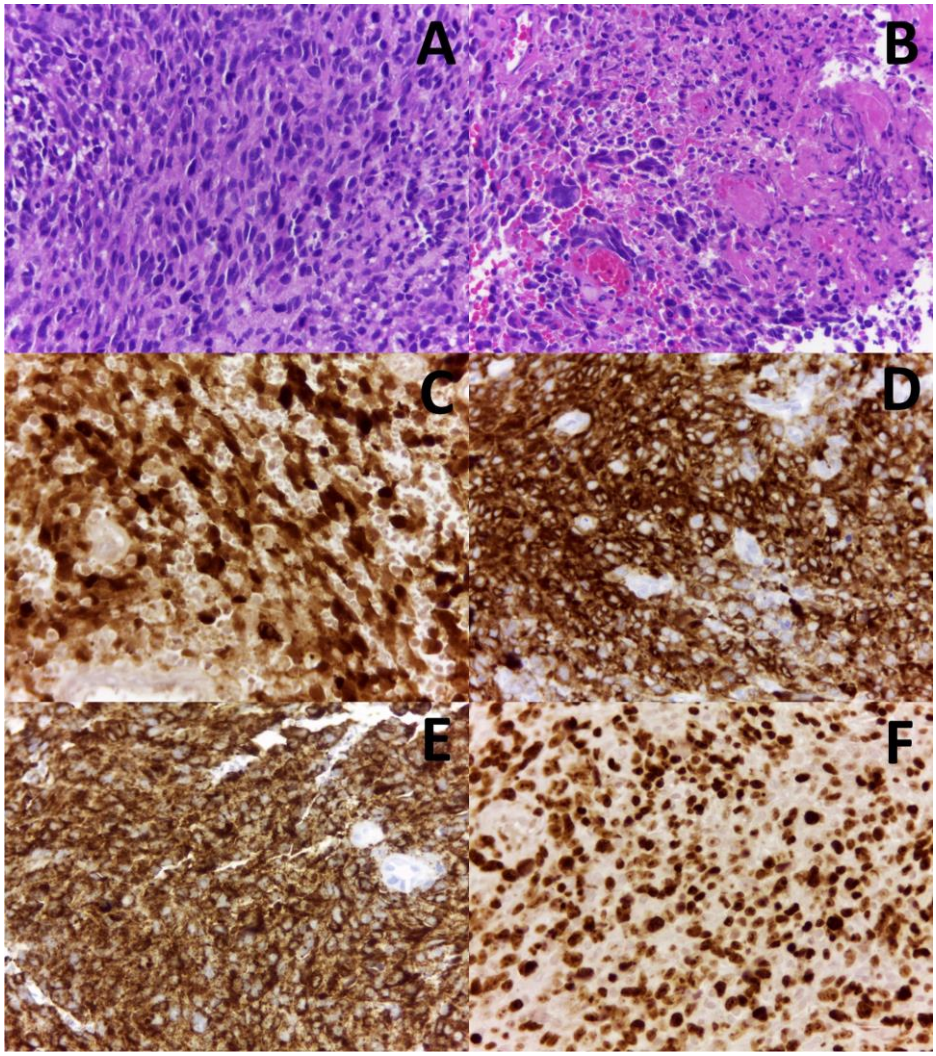

Figure 2. A: Microscopic findings of the case 7, demonstrating a proliferation of spindle and epithelioid melanocytes with a variable number of mitosis and absent or very focal melanin deposition. B: Areas of necrosis and vascular invasion were also observed. The IHC results showed intense immunoreactivity for S-100 (C), MelanA (D), HMB45 (E) and high-proliferative index demonstrated by nuclear Ki67 expression (F).

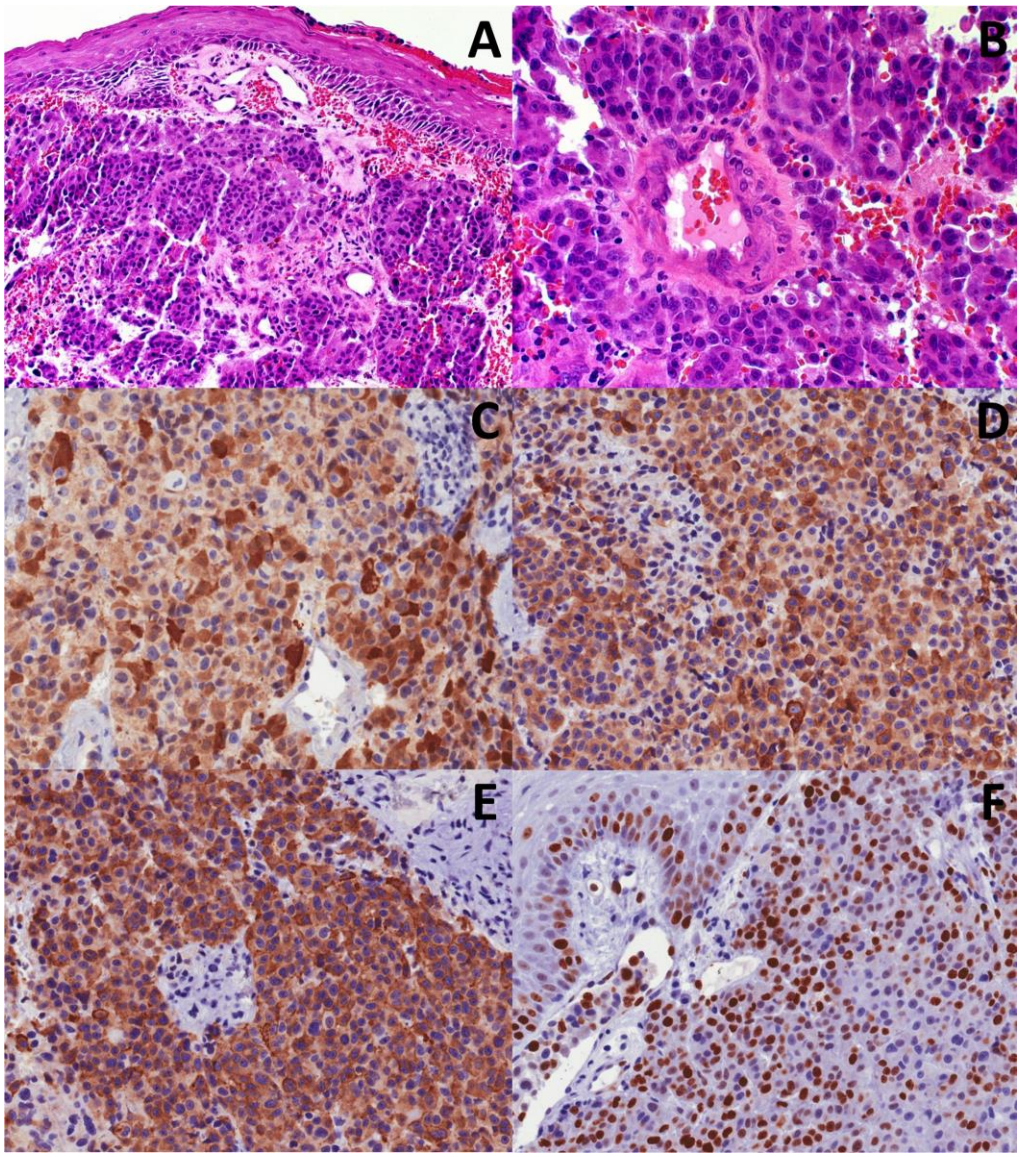

Figure 3. Morphological features of the case 7. A: The tumor was composed predominantly by epithelioid cells arranged in nests; the nuclei were hypercromatic. B: A perivascular pattern was observed in several areas. The IHC results showed intense immunoreactivity for MelanA (C), HMB45 (D), S-100 (E), and very high-proliferative index demonstrated by nuclear Ki67 expression.

Immunohistochemical analysis was subsequently performed for confirmation of the phenotype of tumor cells (Fig. 2 and 3). The results of the immunohistochemical analysis of the evaluated cases are presented in Table 2. The tumor cells were strongly and diffusely positive in all cases for vimentin, protein S-100, and HMB-45. Melan A was considered as focal/weak positive in two cases. In addition, all the cases were negative for smooth muscle actin (α-SMA), pan-cytokeratin AE1/AE3, and anti-leukocyte common antigen (CD45). Ki67-positivity index was >70% in three cases and the case 6 achieved more than 90%.

The patients were treated with surgery and/or chemotherapy (paclitaxel and cisplatin). One of them remains disease-free, 5 died and 2 lost follow-ups (Table 1).

Table 3. Summary of published cases of amelanotic melanomas affecting the oral cavit

Case	Author, year	Age	Gender	Oral Location	Predominant morphology	Immunohistochemical studies	Cervical node metastases	Treatment	Survival from presentation (months)
1	Saku, 1983	59	F	Palate	Desmoplastic	N/A	N/A	N/A	N/A
2	Kumar, 2013	50	F	Tongue	Spindle cells/undifferentiated	CK –, VIM –, desmin–, HMB45+	No	Sx, Rtx	DOD, 6
3	Chu, 1993	79	F	Upper gingiva	Spindle cells/undifferentiated	S100+	Yes	N/A	N/A
4	Boyd, 2011	25	M	Upper gingiva	Small round blue cells	CK–, CD45–, MelanA +, HMB45+,. CD99–, CD138–	Yes	Sx: Chemoterapy (carboplatin, paclitaxel, and pegfilgrastim) and Rtx	DOD, 13
5	Cicconetti, 2009	65	F	Upper gingiva	Spindle cells	CK–,. S-100+, HMB45+	N/A	Sx, Rtx	AWD, 36
6	Pandiar, 2013	50	M	Lower gingiva	Epithelioid/rhabdoid	VIM+, Desmin+, S-100+, Melan-A +, HMB45+, CD68 -, enolase -, myoglobin -	No	Sx, Rtx	N/A
7	Ducic, 2001	68	F	Soft palate	Spindle cells	HMB45+,. S-100+	No	Sx	AWD, 12
8	Godoy, 2009	42	M	Lower lip	Spindle cells	S100+,. HMB45+, CK–	Yes	Sx, Rtx	AWD, N/A
9	Saghravanian, 2014	27	M	Upper gingiva	Epithelioid	S-100+ and HMB45+, LCA–, CD68–, and Desmin–	No	Sx, ChT,	N/A
10	Kao, 2001	80	M	Palate	Undifferentiated/epithelioid	CK–, S100+, HMB45+	No	No treatment	N/A
11	Venugopal, 2013	19	M	Tongue	Epithelioid/spindle cells	Desmin–, VIM+, EMA–, CD34–, HMB45+	Yes	N/A	N/A
12	Moshe, 2018	58	F	Lower gingiva	N/A	N/A	Yes	N/A	DOD, 6
13	Notani, 2002	67	M	Upper gingiva	Spindle cells	S100+, HMB45+	Yes	Sx, ImT (dacarbazine, 1-methyl-3-nitrosourea hydrochloride and vincristine)	DOD, 25
14	Notani, 2002	78	F	Palate	Spindle cells	S100+, HMB45+	Yes	Sx	DOD, 15
15	Notani, 2002	69	M	Retromolar region	Epithelioid	S100+, HMB45+	Yes	Sx, chemotherapy (cisplatin, dacarbazine	AWD, 27

								and vindesine)	
16	Ohnishi, 2015	80	M	Upper gingiva	Epithelioid/Spindle cells	S100+ /.HMB45+ / MelanA +	No	Surgery, ChT: dacarbazine, nimustine and vincristine, and local intracutaneous interferon β therapy (DAV-Feron)	N/A
17	Paulo, 2015	33	F	Lower gingiva	Epithelioid	S100 + / Vimentina +/ HMB45 + / MelanA +	Yes	Sx, ChT	AwD, 60
18	Tanaka, 2004	82	F	Upper gingiva	N/A	S100+, HMB45+	Yes	Rx	DOD, 3
19	Tanaka, 2004	46	M	Upper gingiva	N/A	S100+, HMB45+	Yes	Rtx, ChT, ImT	DOD, 8
20	Tanaka, 2004	71	F	Upper gingiva	N/A	S100+, HMB45+	Yes	Sx, ChT, ImT	DOD, 49
21	Tanaka, 2004	70	F	Upper gingiva	N/A	S100+, HMB45+	No	Sx, ChT, ImT	DOD, 9
22	Tanaka, 2004	84	F	Upper gingiva	N/A	S100+, HMB45+	No	Sx, Rtx	DOD, 29

ChT chemotherapy, Sx Surgery, Rtx Radiotherapy, ImT immunotherapy, AWD alive with disease, AwD alive without disease, DOD died of disease, N/A not available

DISCUSSION

Oral amelanotic melanomas (OAM) are exceedingly rare with less than 35 cases reported in the English literature to date [6–8]. In this study, we present the first large series of eight patients with OAM. Most were male (3:1) and older than 50 years (75%). Four patients presented with relatively advanced disease and three (37.5%) patients had lymph node involvement or metastatic disease at the time of diagnosis. The male predominance in our patients with OAM agrees with several studies [1,3,9]; however, others found a higher predilection of OAM in females [10–12] or no sex predilection [13]. We reviewed 22 cases of OAM with clinical information available. Table 3 lists the demographic and clinical information for these cases. The table does not include eight cases reported by Bachar et al. (2008)[8] and Andrade (2012)[4] because these were series of patients with melanotic and amelanotic oral melanomas and did not have demographic and clinical information about the specific cases. From the data of the reviewed literature, most patients presented neck lymphadenopathy at the time of diagnosis (54.5%), and the majority of the cases occurred in gingiva (59.1%), with only 1 case occurred in soft palate.

Mucosal melanomas of the head and neck region are very aggressive tumors and often are diagnosed at advanced stages [14]. Its occurrence in oral cavity is characterized by detection of pigmented swelling or ulcerated lesion; and microscopically, a proliferation of atypical melanocytes with variable melanin deposition help to establish the diagnosis [4,15]. However, amelanotic tumors present a challenge to clinical and microscopical diagnosis. In fact, the absence of melanin pigmentation makes the clinical diagnosis of these tumors extremely challenging due to the great clinical similarity with several other conditions that affects oral cavity, including reaction lesions, non-neoplastic proliferative processes, infectious diseases, and other oral malignancies. Indeed, microscopically, the difficulty in identifying this differential diagnosis lies in the high variable sarcomatous-like presentation of this lesion, since melanoma can mimic rhabdomyosarcoma, spindle cell carcinoma, lymphomas, poorly differentiated carcinomas, neuroendocrine tumors and poorly differentiated sarcomas. When melanin pigmentation is present, melanomas usually can be diagnosed by morphological analysis. However, when the lesion is amelanotic, the immunohistochemistry analysis is an essential tool for confirming the tumor phenotype and establishing the correct diagnosis. In both situations, positive immunohistochemical reactions for S-100, HMB-45, and Melan-A confirm the diagnosis.

As mentioned above, the differential diagnosis of amelanotic melanomas is very broad because its morphological appearance includes different cell types including epithelioid, spindle and plasmacytoid morphology [16,17]. In our series undifferentiated/epithelioid cells predominated in most cases. Cases with fusiform and rhabdoid predominant morphology were less common. In addition, some other tumors presented a very polymorphous morphology. An extensive IHC panel with markers of neural, neuroendocrine, lymphoid or epithelioid differentiation may be done to rule out other tumors. Neural and vascular invasion were commonly observed in most cases.

Mucosal melanomas frequently show a very diverse morphology, that include fusiform, epithelioid, rhabdoid or even undifferentiated cells. Previous studies have demonstrated no correlation of the type cell with prediction of tumor behavior [18,19]. Nevertheless, some studies have indicated a poor prognosis for tumors with epithelioid morphology [20]. Epithelioid cell type and undifferentiated morphology was markedly prevalent in the amelanotic tumors. These factors may explain the aggressiveness of these tumors; however, other studies can confirm these early observations. According to the review of the literature, epithelioid and spindle cells morphology are the most common presentation for OAM (more than 60%). Rhabdoid and undifferentiated morphology is reported in few cases, representing about less than 9% of all cases.

Similarly to the conventional oral melanoma, OAM presented as masses and/or ulcerated lesions in the gingiva and palate. Only two tumors occurred in other locations, including tongue and lip. The case that occurred in lip presented a non-aggressive course, and the patient remains disease-free 23 months after the surgery. In fact, the intraoral tumors usually show an aggressive behavior probably associated with a very worse prognosis.

Another interesting finding that deserve commentaries is the clinical presentation of OAM in gingiva. These particular cases have clinical diagnosis presumptive of pyogenic granuloma or even other reactive processes. Other series have been reported the possibility of melanomas mimic pyogenic granulomas [1,21]. These factors can be influence in the delayed diagnosis and reinforce the idea that even lesions with a benign clinical presentation must be examined microscopically.

Prasad et al.[2] proposed a microstaging system based in the depth of histologic invasion, considering: level I as exclusive melanoma in situ (very rare for mucosal melanomas), level II as invasion into lamina propria only and level III as deep tissue invasion into adjacent structures including skeletal muscle, bone, or cartilage. All cases

were classified as II/III Prasad-based staging. Our results reinforce the reproducibly of this classification and also confirm that AOM are tumors with particular aggressiveness, indicating a worse outcome for these patients. Several therapeutic options were used to treat mucosal melanomas, including surgery associated or not with chemotherapy/radiotherapy [22]. As particular observation of the authors, it is possible to affirm the management of these tumors remains a challenge and the different therapeutic approach highlight the necessity of standard protocols for oral melanomas.

The prognosis of OAM seems to be extremely poor which may be largely due to the advanced stage of disease at the time of clinical presentation, associated with difficult in stablish an accurate diagnosis [23,24]. Based on previous literature cutaneous amelanotic melanomas have poor prognosis than pigmented ones [5]. So far, only a few cases were reported concerning OAM. In our study, 5 of 8 patients died few months after the first consultation, indicating that oral amelanotic melanomas may have a worse prognosis when compared with the pigmented ones. Overall, the 20% 1-year survival rate in our cohort of OAM (calculated only for patients with follow-up information available) demonstrated the highly aggressive outcome of these tumors that agree with previous report. However, our small sample size did not allow a strong conclusion about this point. Extrapolating from the patients reported in the literature, 45.4% of the patients died with local or disseminated disease (n = 10 of 22 with data), an average of 21.1 months after initial melanoma diagnosis, with 18.2 % of patients alive with local disease (n = 4; mean 25 months follow-up). By contrast, 4.5 % of patients were alive or had died without evidence of disease (n = 1 of 22 with data), with an average follow-up of 27 months. Data were not available for 7 patients.

In conclusion, this study represents one of the largest series of cases of OAM, which seems to exhibit a biological behavior different from the pigmented tumors with metastatic foci present at time of diagnosis. OAM may mimic both clinically and microscopically several benign and malignant asymptomatic conditions of oral cavity, and therefore, immunohistochemical analysis is essential for proper diagnosis, particularly for S-100, HMB-45 and Melan-A. Comparative studies may help to determine specific pathways involved in the pathogenesis of these tumors, as well as to find biological markers to development of targeted therapies.

According to the results of the present cases and previous reports, it is reasonable to suspect that amelanotic melanomas will have an overall poor prognosis because of the aggressiveness of the tumor coupled with a delay in establishing a correct diagnosis.

Early diagnosis by histological examination together with immunochemistry are the keys to improving the survival for patients with oral amelanotic melanoma.

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2.4. Artigo: Comparative expression of cyclooxygenase 2 and Ki67 in amelanotic and conventional oral melanomas

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Running tittle: COX-2 and Ki67 in amelanotic oral melanoma.

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Abstract

Background: Oral melanomas have some histopathological resemblance with its cutaneous counterpart; however, an aggressive behavior is more common in tumors that occur in the oral cavity. Several markers have been suggested as indicative of tumoral progression and aggressiveness, such as cyclooxygenase 2 (COX-2) and Ki67.

Methods: In this study, we have compared the expression of COX-2 and Ki67 in a series of amelanotic (n=7) and melanotic oral melanomas (n=22). The cases were selected from 4 pathology laboratories and submitted to the immunohistochemical (IHC) reactions. We analyzed the IHC staining based on a qualitative – using visual scores; and a computer-assisted method (quantitative) using scanned slides and software for digital analysis.

Results: COX-2 was expressed in all oral melanomas; however, its intensity was significantly higher in the amelanotic ones ($P<0.001$). Similarly, a high Ki67-positivity index was observed in the amelanotic than melanotic ones ($P<0.001$).

Conclusion: Based on these results, we suggest that amelanotic oral melanomas have marked pro-inflammatory and high-proliferative phenotype, justifying their more aggressive behavior compared with the melanotic ones.

Keywords: oral melanoma, amelanotic tumor, cyclooxygenase 2, Ki67

Introduction

Cutaneous and mucosal melanomas origin from cells derivate of the neural crest, the melanocytes.[1] Clinically and microscopically, some tumors do not produce melanin, a brownish pigment that characterize these cells, and are named amelanotic tumors.[2] In the cutaneous counterpart, the absence of melanin is considered a sign of aggressiveness.[3] However, in the tumors of the oral cavity, the significance of this biological event is poorly understood.

Cyclooxygenase 2 (COX-2) is an enzyme involved in several inflammatory pathways. Its main function is the conversion of arachidonic acid to prostaglandin.[4] COX-2 mediate several cellular functions, and in the context of the cancer, its higher expression is associated with modulation of angiogenesis, cellular migration, invasion and proliferation, as well as apoptotic resistance, events that favors tumor progression.[5] In fact, COX-2 is associated with aggressiveness in non-small cell lung cancer[6], pancreatic ductal adenocarcinoma[7], hepatocellular carcinoma[8] and colorectal cancer.[9]

Previous studies of our group have demonstrated that COX-2 expression is associated with angiogenesis in ameloblastoma[10] and cellular proliferation in cutaneous melanomas.[11] However, to date, comparative studies of oral melanotic and amelanotic melanomas are not available in the English language literature. The aim of the present study was to evaluate the immunohistochemical expression of COX-2 and Ki67 in oral melanotic and amelanotic melanomas.

Material and Methods

Formalin-fixed, paraffin-embedded tissue blocks and clinical information of 29 oral melanomas (7 amelanotic and 22 conventional melanotic) were obtained from the charts of four oral pathology laboratories from Latin America (Guatemala, Mexico and 2 from Brazil). Hematoxylin and eosin–stained slides were used for reviewing all diagnoses, and difficult cases were submitted to immunohistochemical analysis (HMB-45, S-100 and MelanA). To classify the melanomas selected in this study, we used a method proposed by Prasad et al.[12].

Accordingly previous protocols used in our laboratory, for immunohistochemical staining, 3-µm-thick sections were deparaffinized, rehydrated in graded ethanol solutions and after antigen retrieval with EDTA/Tris buffer (pH 9.0) in a pressure cooker; endogenous peroxidase activity was blocked with 20% H2O2 during 15 min. We used a method of 2-hours incubation with the primary antibodies: COX-2 (Clone: CX-294, dilution 1:100; Dako Corporation, Carpinteria, CA, USA) and Ki67 (Clone: MIB-1, dilution 1:100; Dako Corporation). Both diluted in BSA (bovine serum albumin). The secondary antibody conjugated with polymer dextran marked with peroxidase (Dako EnVision Labelled Polymer; Dako, Glostrup, Denmark) was applied for 1 hour. The reaction was developed with Permanent Red (Permanent Red Substrate System; Dako) and counterstained with Carazzi hematoxylin. Sections of oral squamous cell carcinoma were included in all reactions as positive control for both markers. Negative controls of reactions were performed by omitting the primary antibody. Only cytoplasmic staining was considered as positive for COX-2, and only nuclear reactivity was considered for Ki67. The quantification of the immunoreactivity was determined using a digital method previously validated by our group.[10,13]

Results

Amelanotic melanomas were more common in men (5 males, 2 females), with a mean age of 51.5 years. The most common location was gingiva (3 cases), followed by palate (2 cases) and tongue and lip with one case. Five patients died from complications of the disease, 1 lost follow-up and 1 is disease-free. All but one patient was treated with surgery and complementary chemotherapy.

The conventional melanotic melanomas occurred predominantly in women (14 females, 8 males), with a mean age of 58 years. The locations were palate (n=14), gingiva (n=4) and other anatomical sites (n=4). Regarding treatment, 15 were treated with only surgery and 7 received complementary chemotherapy. Twelve patients died from complications of the tumor and 10 are alive.

COX-2 was expressed in the cytoplasm of tumor cells and occasionally in inflammatory cells. All melanomas were positive for COX-2 in different degrees. In the present series COX-2 expression had the highest positivity index for amelanotic (scores ranging from 143 to 294, mean 206), followed by melanotic melanomas (scores ranging from 101 to 144, mean 122.5). Representative photomicrographs are illustrated in the Figure 1. The means were statistically different (t test, P<0.001).

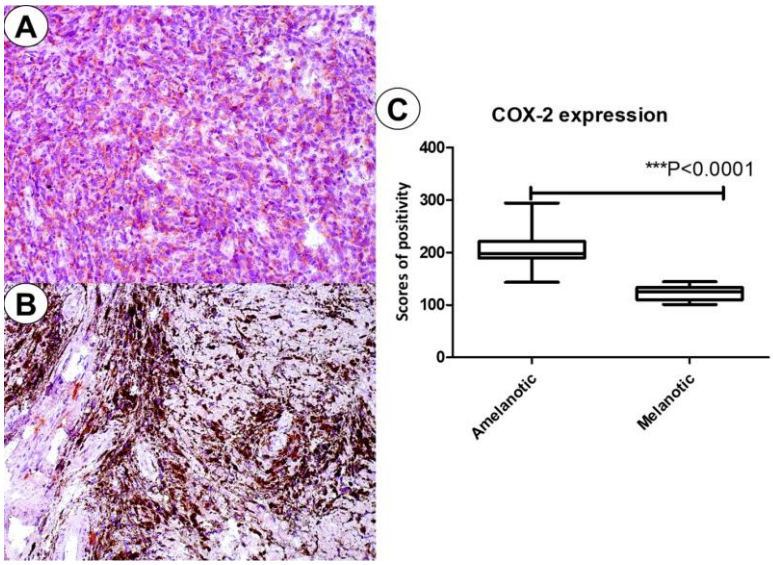

Figure 1. COX-2 expression in amelanotic (A) and melanotic melanomas (B). Graphical representation of the scores of immunopositivity.

Ki-67 presented a nuclear expression mainly in tumor cells. The index was digitally calculated and were higher for amelanotic (ranging from 34 to 92%, mean 64%) than melanotic melanomas (ranging from 11 to 59%, mean 30.8%). These results are described in the Figure 2. The means were statistically different (t test, P<0.001).

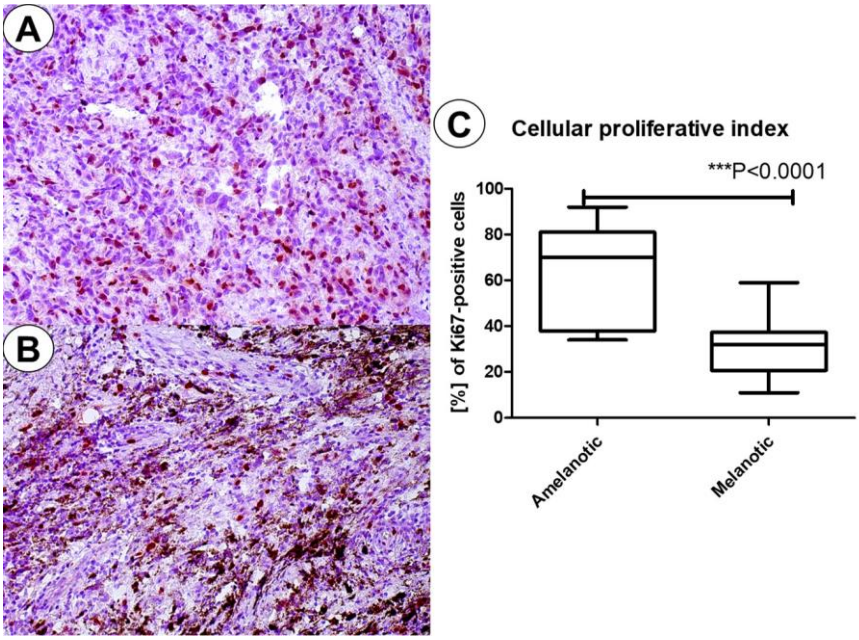

Figure 2. Ki67-nuclear expression in amelanotic (A) and melanotic melanomas (B). Graphical representation of the cellular proliferative index based on Ki67 expression.

Discussion

To the best of our knowledge this is the first study to compare the expression of COX-2 and Ki67 in amelanotic versus conventional melanotic oral melanomas. In the present study we found that both studied markers demonstrated higher expression in amelanotic melanomas than melanotic ones. The main theory for these results, is that the amelanotic tumors represent a most undifferentiated neoplasm, and the presence of melanin may represent a more differentiated phenotype. Other theory is that amelanotic tumors represent a de-differentiated conventional melanoma. In our opinion, it is very difficult to affirm which theory is more adequate. We only can confirm that amelanotic tumors really present a more aggressive behavior.

Tumor cell proliferation index is widely recognized to indicate the degree of aggressiveness of a tumor. In addition, Ki67-index has been used as prognostic factor for several tumors, including breast cancer[14] and mucosal melanomas.[15] Although our data suggest a relationship between COX-2 expression and higher cell proliferation, other features must be considered including the idea that amelanotic tumors are more undifferentiated, have higher frequency of necrosis and mitosis than melanotic melanomas.

In fact, the higher expression of COX-2 in amelanotic corroborates with the hypothesis that these tumors have a poorer clinical prognosis, once COX-2 is involved

in several pro-tumorigenic events, including angiogenesis[7], cell proliferation and migration[6] and association with a metastatic phenotype.[11] Additional studies are necessary to determine the biological differences between amelanotic and melanotic oral melanomas.

In the cutaneous melanomas, the absence of melanin is an indicator of aggressiveness. Cheung et al.[3] have demonstrated that a higher mitotic index in amelanotic melanoma correlates with greater nuclear atypia and a worse prognosis. Similarly, our results demonstrated that oral amelanotic melanomas have a higher Ki67-index than the pigmented ones.

In summary, amelanotic melanomas had a higher COX-2 expression and higher cellular proliferative than melanotic ones. These data suggest that amelanotic melanomas differ from the melanotic subtype and more studies are necessary to better understand the role that the absence of melanin deposition may cause in the tumoral biology. It is important to keep in mind that, due to the rarity of these neoplasms, the data presented here must be interpreted with caution.

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2.5 Artigo: Prognostic importance of mitochondrial markers in head and neck mucosal and cutaneous melanomas

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Running Head: Mitochondrial markers predict survival in melanomas

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ABSTRACT

Mitochondrial dysfunction is caused by an imbalance in the processes of fission and fusion, and it has been implicated in the pathogenesis of several human cancers. However, the role of mitochondrial markers in melanomas still remain poorly understood. In this study, we assessed the expression of three mitochondrial markers [antimitochondrial (AMT), fission protein 1 (FIS1) and mitofusin 2 (MFN2)] in a series of head and neck mucosal and cutaneous melanoma (CM). One hundred and twelve patients with cutaneous (n=56), and mucosal [oral – n=30 and sinonasal – n=26] melanomas of the head and neck region were enrolled in this study. Clinical and follow-up data were retrieved from the medical records. The expression of three mitochondrial markers was assessed by the immunohistochemistry, digitally quantified and correlated with clinicopathological data and outcome information. In the multivariate model, high mitochondrial content was identified as an independent prognostic value for disease-free survival (DFS) in cutaneous melanomas and overall survival (OS) in oral melanomas. FIS1 expression was significantly associated with lower OS rates in patients with oral melanomas, and strictly correlated with vascular invasion in mucosal melanomas. MFN2 was identified as an independent prognostic marker for DFS and OS in patients with CM, and it was associated with a high risk of distant metastasis in these tumors. In summary, we demonstrated that mitochondrial content and FIS1 and MFN2 expressions are correlated with important clinicopathologic characteristics in patients with cutaneous and mucosal melanomas of the head and neck region.

Keywords: head and neck melanomas, cutaneous melanomas, mucosal melanomas, mitochondria, FIS1, MFN2, prognosis.

INTRODUCTION

Mitochondria are highly dynamic organelles involved in the energy production of all human cells [1]. Several biological processes require the ATP as an energy source through oxidative phosphorylation (OXPHOS) [2]. As a consequence of the OXPHOS, mitochondria also produce the majority of reactive oxygen species (ROS). ROS are implicated in the carcinogenic process, damaging important proteins and macromolecules, causing a deregulation in the cell cycle and other relevant process for the tumor development and metastasis [3]. In addition, mitochondria play an essential role in apoptosis, regulating critical signaling pathways for its activation or inhibition [4].

Currently, there has been described the predictive value of mitochondrial markers in recurrence, metastasis and tamoxifen-resistance of breast cancer patients [5]. In the intracellular compartment, mitochondria constantly change its number and shape in an event named mitochondrial dynamics, which involves two main processes: fusion and fission [1]. Although these processes are widely investigated, their role in cancer cells and consequently in the context of clinical behavior, tumorigenesis and metastasis remains poorly studied in melanoma.

Melanoma is an aggressive tumor that arises most commonly on the skin or mucous membrane. Prognosis of primary melanoma is based on morphological parameters such as tumor thickness and mitotic index [6,7]. Considering that cutaneous melanomas (CM) is strictly associated with sun exposure, whereas in mucosal melanomas (MM) it has no effect in their pathogenesis, comparative studies are necessary to clarify the differences in signaling pathways involved in the different subtypes of melanomas [8,9]. In addition, there is currently demonstrated that CM and MM harbor different genetic alterations and few studies have compared mucosal and

cutaneous melanomas of the head and neck (H&N) region [10]. Oral and sinonasal melanomas are uncommon tumors that arise in the mucosa of these anatomical sites, presenting an aggressive behavior, tendency to metastasize and consequently worse prognosis than cutaneous melanomas [11,12]. To date, few studies have attempted to describe proteins with prognostic predictive value for these tumors.

The role of metabolic markers in several cancers is well stablished [13–15]. Moreover, high levels of ROS are associated with tumor development and progression, and as the main source of ROS are mitochondria, we hypothesized that these organelles might play an essential role in tumorigenesis and impacts in the biological behavior of several cancers [16]. However, in melanomas, it is not fully clarified. In the current study, we compare the expression of three mitochondrial markers in oral, sinonasal and cutaneous melanomas of the H&N region.

METHODS

Case selection

We retrospectively collected 112 H&N cutaneous and mucosal melanoma cases with follow-up and complete clinical information from four pathology laboratories. Formalin-fixed, paraffin-embedded tissue blocks were retrieved; the diagnoses of the melanomas were reviewed and confirmed by three pathologists. In addition, we performed S-100, MelanA and HMB45 immunostainings to all cases.

Immunohistochemistry

For immunohistochemical (IHC) polymer-based method, 3-µm-thick sections mounted on silanized slides were used. The sections were deparaffinized, rehydrated in graded ethanol solutions and submitted to antigen retrieval with EDTA/Tris buffer (pH

9.0) in an electric pressure cooker for 15 minutes. Following that, endogenous peroxidase activity was blocked with 20% H2O2 by single incubation of 15 minutes.

The sections were then incubated with the diluted primary antibodies for two hours at room temperature. We used two high-sensitive visualization systems: ADVANCE™/HRP (code K406889-2; Dako, Carpinteria, CA, USA) and EnVision G|2 System/AP, Rabbit/Mouse (Permanent Red) (code K535521-2, Dako). The IHQ reactions were revealed with Permanent Red (Dako) or DAB (Sigma Aldrich, St. Louis, MO, USA) and counterstained with Carazzi's hematoxylin. Detailed information about the primary antibodies, dilution, manufacturer and respective positive controls is listed in Supplementary Table 1.

Digital analysis

Following the IHC reactions, the slides were scanned into high-resolution images and digitally assessed as described previously with the scores of positivity ranged from 100 (very weak) to 300 (strongly positive) [17]. One calibrated pathologist selected 10 different regions per case to assess the digital IHC quantification, these areas were from the superior and inferior portions of the tumors and not included highly melanin-pigmented areas.

Statistical methods

Briefly, for the statistical methods, we divided the cutaneous melanomas into two groups based in the Clark's level: (1) I, II and III and (2) IV and V. The scores of immunostaining were correlated with clinical data from all types of melanomas using contingency tables and Chi-Square or Fisher's exact test. Survival curves were calculated according to the Kaplan-Meier method. The Log-Rank test was applied for patients with low and high expression of the markers (cutoff value: median of the scores

of positivity). COX proportional hazards regression analysis was performed to test the statistical independence and significance between pathological, molecular and clinical variables. All statistical tests were carried out in the SPSS software, version 22.0 (SPSS Inc., Chicago, IL, USA) with a 95% confidence level (P-value ≤ 0.05).

The National Commission for Ethics in Research approved the study protocol (CONEP-Brazil, CAAE: 72077517.1.0000.5418). This study was conducted according to the Declaration of Helsinki Principles.

RESULTS

The immunostaining scores were quantitatively assessed by digital analysis. All mitochondrial markers demonstrated a high expression in tumor cells. Overall, expression of AMT was higher in mucosal than in cutaneous melanomas (Figure 1); FIS1 immunostaining was high in oral melanomas and closely correlated with tumor cells in areas of vascular and neural invasion (Figure 2) and expression of MFN2 was very higher in cutaneous and sinonasal melanomas than in oral melanomas (Figure 3). The median scores for cutaneous melanomas were 199.4 (range 248.8–118.4 for AMT), 134.5 (range 175.9–101.9 for FIS1) and 211.9 (range 255.7–156.2 for MFN2). For oral melanomas, 224.4 (range 268.1–109.3 for AMT), 179.7 (range 230.0–116.1 for FIS1) and 182.7 (range 226.4–142.0 for MFN2). For sinonasal melanomas, 229.4 (range 258.9–173.5 for AMT), 124.1 (range 181.0–112.3 for FIS1) and 239.8 (range 259.4–208.7 for MFN2).

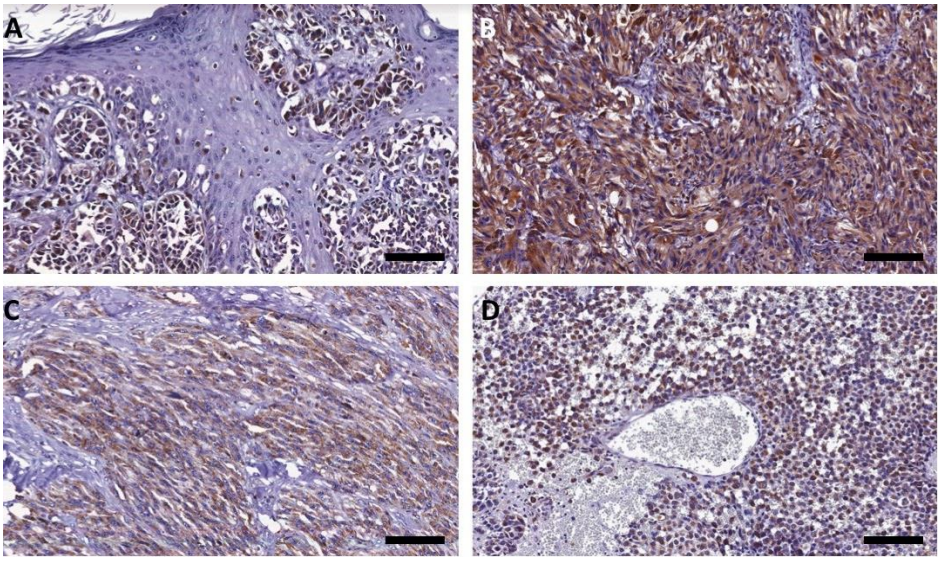

Figure 1. AMT expression in cutaneous and mucosal head and neck melanomas. A: Medium cytoplasmic positivity in malignant melanocytes in cutaneous melanoma. B: Strong positivity in a predominant fusiform oral melanoma. C: Strong positivity in a spindle/epithelioid sinonasal melanoma. D: Perinuclear pattern of AMT immunostaining in sinonasal melanomas, undifferentiated type. The magnification scale is 100 μm for all figures.

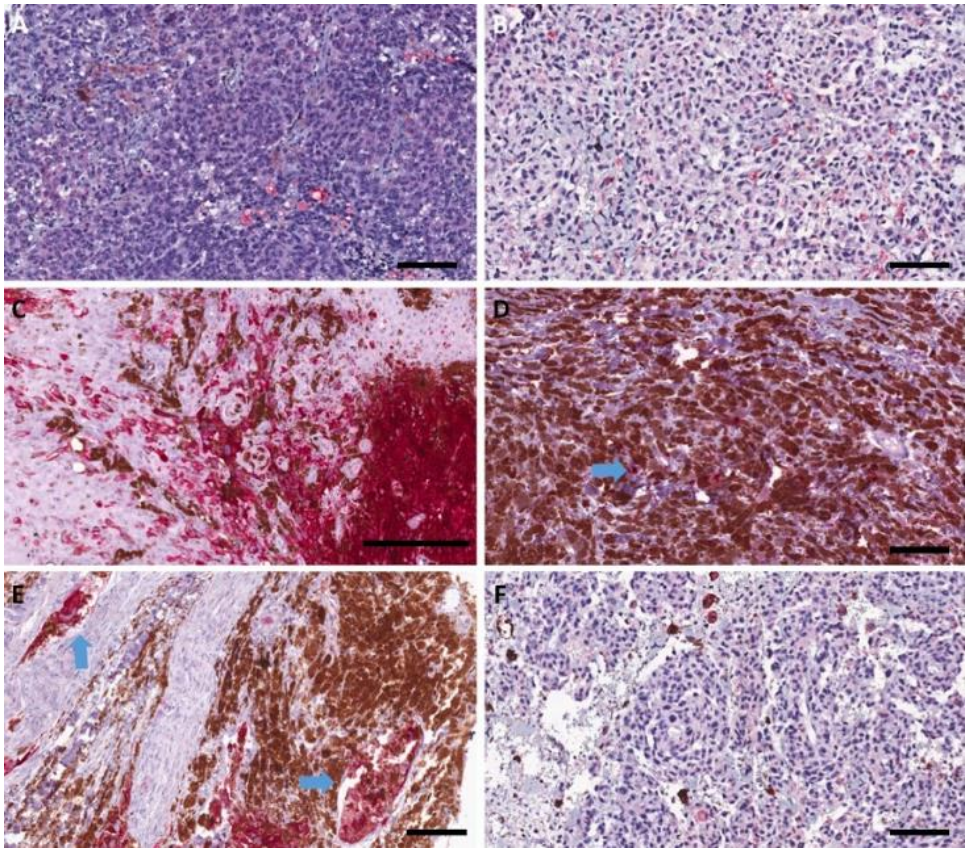

Figure 2. FIS1 expression in cutaneous and mucosal head and neck melanomas. A: Medium cytoplasmic positivity in macrophages, and very weak FIS1 expression in cutaneous melanoma. B: Weak cytoplasmic expression in tumor cells of cutaneous melanoma. C: Strong FIS1 expression in malignant melanocytes of oral melanoma. D: FIS1-positive cells during mitosis (blue arrows). E: Strong FIS1 positivity in vascular and neural invasion areas. F: Weak FIS1 immunostaining in tumor cells of sinonasal melanomas,

occasional macrophages are positive. The magnification scale is 100 µm for all figures, except for C, which represents 200 µm.

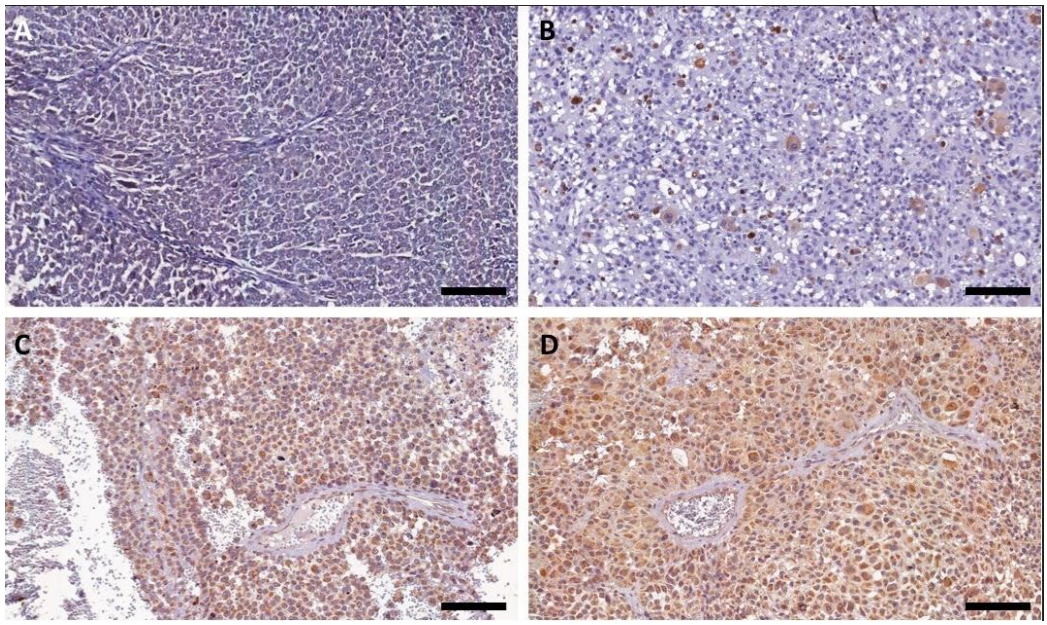

Figure 3. MFN2 expression in cutaneous and mucosal head and neck melanomas. A: Medium MFN2 immunostaining in cutaneous melanoma. B: Positivity of MFN2 was focal and in individual cells of oral melanomas. C: Perinuclear immunopositivity of MFN2 was observed in some cases of sinonasal melanomas. D: Strong positivity of MFN2 in a case of sinonasal melanoma (stage IVc). The magnification scale is 100 µm for all figures.

Cutaneous melanomas

A total of fifty-six patients with cutaneous melanomas of the head and neck region (25 female, 31 male) were included. The cases distribution of AJCC-stages was as follows: stage 0, In situ 5.4% (3/56), IA 17.9% (10/56), IB 8.8% (5/56), IIA 10.7% (6/56), IIB 12.5% (7/56), IIC 8.9% (5/56), IIIA 5.4% (3/56), IIIB 5.4% (3/56), IIIC 7.1% (4/56) and IV 17.9% (10/56). The mean of Breslow's thickness was 3.0 mm (ranging from 0 to 38 mm).

All mitochondrial markers studied here demonstrated a correlation with the presence of ulceration, Breslow's thickness and mitotic index. AMT and FIS1 were associated with AJCC-stage, whereas FIS1 and MFN2 were correlated with Clark's level. High expression of FIS1 was also correlated with vertical growth phase (Table 1).

Table 1. Relationship of AMT, FIS1, and MFN2 expressions with clinicopathological characteristics of all patients with HN cutaneous melanomas (n=56).

		AMT [n (%)]		P-value	FIS1 [n (%)]		P-value	MFN2 [n (%)]		P-value
Variables	Categories	Low	High		Low	High		Low	High	
Age	<56	14 (51.9)	15 (51.7)	0.9923	14 (50.0)	15 (53.6)	0.7891	13 (46.4)	16 (57.1)	0.4223
	≥56	13 (48.1)	14 (48.3)		14 (50.0)	13 (46.4)		15 (53.6)	12 (42.9)	
Sex	Female	13 (48.1)	12 (41.4)	0.6106	13 (46.4)	12 (42.9)	0.7880	13 (46.4)	12 (42.9)	0.7880
	Male	14 (51.9)	17 (58.6)		15 (53.6)	16 (57.1)		15 (53.6)	16 (57.1)	
Ulceration	Absent	21 (77.8)	13 (44.8)	0.0116*	24 (85.7)	10 (35.7)	0.0001*	24 (85.7)	10 (35.7)	0.0001*
	Present	6 (22.2)	16 (55.2)		4 (14.3)	18 (64.3)		4 (14.3)	18 (64.3)	
Growth phase	Radial	10 (37.0)	12 (41.4)	0.7395	15 (53.6)	7 (25.0)	0.0286*	13 (46.4)	9 (32.1)	0.2737
	Vertical	17 (63.0)	17 (58.6)		13 (47.4)	21 (75.0)		15 (53.6)	19 (67.9)	
Breslow's thickness (mm)	<3.3	23 (85.2)	16 (55.2)	0.0146*	25 (89.3)	14 (50.0)	0.0013*	24 (85.7)	15 (53.6)	0.0089*
	≥3.3	4 (14.8)	13 (44.8)		3 (10.7)	14 (50.0)		4 (14.3)	13 (46.4)	
Clark's level	I, II, III	15 (55.6)	10 (34.5)	0.1129	22 (78.6)	3 (10.7)	<0.0001*	17 (60.7)	8 (28.6)	0.0155*
	IV, V	12 (44.4)	19 (65.5)		6 (21.4)	25 (89.3)		11 (39.3)	20 (71.4)	
Mitotic index (mm^2)	<3	16 (59.3)	8 (27.6)	0.0167*	19 (67.9)	5 (17.9)	0.0001*	19 (67.9)	5 (17.9)	0.0001*
	≥3	11 (40.7)	21 (72.4)		9 (32.1)	23 (82.1)		9 (32.1)	23 (82.1)	
AJCC-stage	I and II	24 (88.9)	9 (31.0)	<0.0001*	19 (67.9)	14 (50.0)	0.1744	24 (85.7)	9 (32.1)	<0.0001*
	III and IV	3 (11.1)	20 (69.0)		9 (32.1)	14 (50.0)		4 (14.3)	19 (67.9)	

AMT, mitochondrial antibody; FIS1, fission 1 protein; MFN2, mitofusin 2; HN, Head and Neck; AJCC, American Joint Committee on Cancer. Asterisks (*) indicate statistical significance (P<0.05).

Table 2. Relationship of clinicopathological characteristics of patients with HN cutaneous melanomas (n=56) and AMT, FIS1, and MFN2 expressions with DFS and OS rates in univariate and multivariate models.

Variables	**Categories**		DFS univariate (%)		P-value (log-rank)	Multivariate		OS univariate (%)		P-value (log-rank)	Multivariate	
		n (%)	3-years	5-years		HR (95% CI)	*P*-value	3-years	5-years		HR (95% CI)	P-value
Age	<56	29 (51.8)	93.1	77.3	0.29	–	–	93.1	81.6	0.21	–	–
	≥56	27 (48.2)	92.3	59.9	(1.09)			92.3	67.6	(1.57)		
Sex	Female	25 (44.6)	95.7	77.0	0.39	–	–	95.7	82.2	0.22	–	–
	Male	31 (55.4)	90.3	62.0	(0.71)			90.3	68.9	(1.46)		
Ulceration	Absent	34 (60.7)	97.1	87.4	<0.01*	3.27	0.08	97.1	90.9	<0.01*	2.23	0.34
	Present	22 (39.3)	85.4	30.1	(24.17)	(0.86 – 12.35)		85.4	45.4	(14.76)	(0.42 – 11.76)	
Growth phase	Radial	22 (39.3)	95.5	71.3	0.47	–	–	95.5	76.4	0.24	–	–
	Vertical	34 (60.7)	87.7	67.5	(0.51)			87.8	74.4	(1.34)		
Breslow's	<3.3	39 (69.5)	97.4	86.4	<0.01*	4.61	0.02*	97.4	86.4	<0.01*	4.593	0.052
thickness (mm)	≥3.3	17 (30.5)	81.6	30.1	(13.43)	(1.24 – 17.11)		81.6	48.3	(7.69)	(0.98 – 21.43)	
Clark's level	I, II, III	25 (44.6)	95.8	77.9	0.02*	4.32	0.44	100.0	82.8	0.23	–	–
	IV, V	31 (55.4)	86.6	57.0	(4.79)	(0.1 – 184.66)		86.6	68.2	(1.40)		
Mitotic index	<3	24 (42.8)	95.8	91.7	<0.01*	5.168	0.04*	95.8	91.7	<0.01*	2.87	0.20
(mm^2)	≥3	32 (57.2)	90.2	50.9	(16.32)	(1.06 – 24.98)		90.2	61.1	(7.84)	(0.55 – 14.82)	
AJCC-stage	I and II	33 (58.9)	96.6	85.5	<0.01*	2.469	0.18	100.0	96.4	<0.01*	11.21	0.03*
	III and IV	23 (41.1)	82.6	41.1	(24.48)	(0.65 – 9.343)		82.6	46.2	(22.48)	(1.18 – 106.003)	
AMT	Low	27 (48.2)	100.0	95.5	<0.01*	24.07	0.012*	100.0	95.5	<0.01*	1.32	0.79
	High	29 (51.8)	85.9	46.0	(26.72)	(2.03 – 284.66)		85.9	56.5	(12.67)	(0.15 – 11.34)	
FIS1	Low	28 (50.0)	96.4	81.3	<0.01*	0.04	0.14	96.4	85.6	0.06	0.24	0.13
	High	28 (50.0)	88.7	49.8	(8.92)	(0.001 – 2.989)		88.7	62.5	(3.32)	(0.041 – 1.52)	
MFN2	Low	28 (50.0)	100.0	88.7	<0.01*	1.14	0.87	100.0	92.7	<0.01*	2.09	0.49
	High	28 (50.0)	85.1	45.9	24.03	(0.21 – 6.181)		85.1	53.7	(15.06)	(0.25 – 17.31)	

AMT, mitochondrial antibody; FIS1, fission 1 protein; MFN2, mitofusin 2; HN, Head and Neck; AJCC, American Joint Committee on Cancer; DFS, Disease-free survival; OS, overall survival, HR, Harzard Ratio; CI, Confidence interval.
Asterisks (*) indicate statistical significance (P<0.05).

Table 3. Relationship of AMT, FIS1, and MFN2 expressions with clinicopathological characteristics of all patients with oral melanomas (n=30).

		AMT [n (%)]		P-value	FIS1 [n (%)]		P-value	MFN2 [n (%)]		P-value
Variables	Categories	Low	High		Low	High		Low	High	
Age	<48	6 (40.0)	10 (66.7)	0.1432	7 (46.7)	9 (60.0)	0.4642	7 (46.7)	9 (60.0)	0.4642
	≥48	9 (60.0)	5 (33.3)		8 (53.3)	6 (40.0)		8 (53.3)	6 (40.0)	
Sex	Female	9 (60.0)	7 (46.7)	0.4642	6 (40.0)	10 (66.7)	0.1432	10 (66.7)	6 (40.0)	0.1432
	Male	6 (40.0)	8 (53.3)		9 (60.0)	5 (33.3)		5 (33.3)	9 (60.0)	
Site	Palate	6 (40.0)	7 (46.7)	0.8706	5 (33.3)	8 (53.3)	0.0641	8 (53.3)	5 (33.3)	0.5418
	Gingiva	3 (20.0)	2 (13.3)		1 (6.7)	4 (26.7)		2 (13.3)	3 (20.0)	
	Others	6 (40.0)	6 (40.0)		9 (60.0)	3 (20.0)		5 (33.4)	7 (46.7)	
Treatment	Only Surgery	12 (80.0)	8 (53.2)	0.2465	12 (80.0)	8 (53.3)	0.2465	9 (60.0)	11 (73.3)	0.5191
	Surgery plus CH/RT	3 (20.0)	6 (40.0)		3 (20.0)	6 (40.0)		5 (33.3)	4 (26.7)	
	No treatment	0 (0.0)	1 (6.7)		0 (0.0)	1 (6.7)		1 (6.7)	0 (0.0)	
Vascular invasion	Absent	10 (66.7)	9 (60.0)	0.7047	15 (100)	4 (26.7)	<0.001*	9 (60.0)	10 (66.7)	0.7047
	Present	5 (33.3)	6 (40.0)		0 (0.0)	11 (73.3)		6 (40.0)	5 (33.3)	
Neural invasion	Absent	13 (86.7)	11 (73.3)	0.3613	14 (93.3)	10 (66.7)	0.0678	12 (80.0)	12 (80.0)	1.000
	Present	2 (13.3)	4 (26.7)		1 (6.7)	5 (33.3)		3 (20.0)	3 (20.0)	
Mitotic index	<1	9 (60.0)	4 (26.7)	0.0654	11 (73.3)	2 (13.3)	<0.001*	6 (40.0)	7 (46.7)	0.7125
	≥1	6 (40.0)	11 (73.3)		4 (26.7)	13 (86.7)		9 (60.0)	8 (53.3)	
Cellular morphology	Non-epithelioid	8 (53.3)	2 (13.3)	0.0201*	7 (46.7)	3 (20.0)	0.1213	5 (33.3)	5 (33.3)	1.000
	Epithelioid	7 (46.7)	13 (86.7)		8 (53.3)	12 (80.0)		10 (66.7)	10 (66.7)	
AJCC-stage	III	8 (53.3)	5 (33.3)	0.4843	8 (53.3)	5 (33.3)	0.0233*	8 (53.3)	5 (33.3)	0.0201*
	IVa	5 (33.3)	6 (40.0)		7 (46.7)	4 (26.7)		2 (13.4)	9 (60.0)	
	IVb and IVc	2 (13.4)	4 (26.7)		0 (0.0)	6 (40.0)		5 (33.3)	1 (6.7)	

AMT, mitochondrial antibody; FIS1, fission 1 protein; MFN2, mitofusin 2; AJCC, American Joint Committee on Cancer; CH, chemotherapy; RT, radiotherapy.
Asterisks (*) indicate statistical significance (P<0.05).

Table 4. Relationship of clinicopathological characteristics of patients with oral melanomas (n=30) and AMT, FIS1, and MFN2 expressions with OS rates in univariate and multivariate models.

Variables	**Categories**		OS univariate (%)		P-value (log-rank)	Multivariate	
		n (%)	3-years	5-years		HR (95% CI)	P-value
Age	<48	16 (53.3)	56.3	40.2	0.76	–	–
	≥48	14 (46.7)	43.8	43.8	(0.09)		
Sex	Female	16 (53.3)	38.4	38.4	0.33	–	–
	Male	14 (46.7)	64.3	42.9	(0.94)		
Anatomical site	Palate	13 (43.3)	27.7	0.0	0.02*	0.33	0.02*
	Gingiva	5 (16.6)	40.0	40.0	(7.46)	(0.13 – 0.85)	
	Others	12 (40.1)	81.8	65.5			
Treatment	Only surgery	20 (66.7)	57.4	57.4	0.21	–	–
	Surgery plus CH/RT	9 (30.0)	44.4	14.8	(1.57)		
	No treatment	1 (3.3)	–	–			
Vascular invasion	Absent	19 (63.3)	83.6	61.0	<0.01*	1.61	0.69
	Present	11 (36.7)	0.0	0.0	(20.69)	(0.14 – 17.97)	
Neural invasion	Absent	24 (80.0)	60.4	44.1	0.03*	0.82	0.81
	Present	6 (20.0)	16.7	16.7	(4.48)	(0.17 – 3.93)	
Mitotic index (mm^2)	<1	13 (43.3)	91.7	91.7	<0.01*	6.75	0.18
	≥1	17 (56.7)	23.5	11.7	(13.93)	(0.42 – 109.16)	
Necrosis	Absent	22 (73.3)	51.3	41.0	0.59	–	–
	Present	8 (26.7)	50.0	25.0	(0.28)		
Cellular morphology	Non-epithelioid	10 (33.3)	88.9	88.9	<0.01*	8.56	0.11
	Epithelioid	20 (66.7)	35.0	0.0	(9.76)	(0.59 – 122.71)	
AJCC–stage	III	13 (43.3)	68.4	51.3	<0.01*	1.25	0.61
	IVa	11 (36.7)	60.6	40.4	(14.66)	(0.52 – 3.05)	
	IVb and IVc	6 (20.0)	0.0	0.0			
AMT	Low	15 (50.0)	62.7	62.7	0.03*	12.97	0.01*
	High	15 (50.0)	40.0	13.3	(4.48)	(1.73 – 97.09)	
FIS1	Low	15 (50.0)	92.9	63.7	<0.01*	22.99	0.03*
	High	15 (50.0)	13.3	13.3	(15.45)	(1.32 – 401.12)	
MFN2	Low	15 (50.0)	52.5	28.0	0.49	–	–
	High	15 (50.0)	50.3	50.3	(0.47)		

AMT, mitochondrial antibody; FIS1, fission 1 protein; MFN2, mitofusin 2; AJCC, American Joint Committee on Cancer; OS, overall survival, HR, Harzard Ratio; CI, Confidence interval.
Asterisks (*) indicate statistical significance (P<0.05).

Table 5. Relationship of AMT, FIS1, and MFN2 expressions with clinicopathological characteristics of all patients with sinonasal melanomas (n=26).

		AMT [n (%)]		P-value	FIS1 [n (%)]		P-value	MFN2 [n (%)]		P-value
Variables	Categories	Low	High		Low	High		Low	High	
Age	<59	6 (46.2)	7 (53.8)	0.6948	7 (53.8)	6 (46.2)	0.6948	4 (30.8)	9 (69.2)	0.0498*
	≥59	7 (53.8)	6 (46.2)		6 (46.2)	7 (53.8)		9 (69.2)	4 (30.8)	
Sex	Female	6 (46.2)	8 (61.5)	0.4314	8 (61.5)	6 (46.2)	0.4314	10 (76.9)	4 (30.8)	0.0182*
	Male	7 (53.8)	5 (38.5)		5 (38.5)	7 (53.8)		3 (23.1)	9 (69.2)	
Site	Nasal cavity	6 (46.2)	5 (38.4)	0.3180	7 (53.8)	4 (30.8)	0.4920	4 (30.8)	7 (53.8)	0.4920
	Paranasal sinuses	6 (46.2)	4 (30.8)		4 (30.8)	6 (46.2)		6 (46.1)	4 (30.8)	
	Rinopharynx	1 (7.6)	4 (30.8)		2 (15.4)	3 (23.0)		3 (23.1)	2 (15.2)	
Treatment	Only Surgery	6 (46.2)	5 (38.5)	0.5024	7 (53.8)	4 (30.8)	0.3492	5 (38.5)	6 (46.2)	0.5024
	Surgery plus CH/RT	6 (46.2)	8 (61.5)		6 (46.2)	8 (61.5)		8 (61.5)	6 (46.2)	
	No treatment	1 (7.6)	0 (0.0)		0 (0.0)	1 (7.7)		0 (0.0)	1 (7.6)	
Vascular invasion	Absent	10 (76.9)	9 (69.2)	0.6583	12 (92.3)	7 (53.8)	0.0270*	11 (84.7)	8 (61.5)	0.1847
	Present	3 (23.1)	4 (30.8)		1 (7.7)	6 (46.2)		2 (15.3)	5 (38.5)	
Neural invasion	Absent	11 (84.6)	13 (100)	0.1410	13 (100)	11 (84.7)	0.1410	13 (100)	11 (84.7)	0.1410
	Present	2 (15.4)	0 (0.0)		0 (0.0)	2 (15.3)		0 (0.0)	2 (15.3)	
Mitotic index	<1	10 (76.9)	8 (61.5)	0.3954	10 (76.9)	8 (61.5)	0.3954	8 (61.5)	10 (76.9)	0.3954
	≥1	3 (23.1)	5 (38.5)		3 (23.1)	5 (38.5)		5 (38.5)	3 (23.1)	
Cellular morphology	Plasmacytoid/Others	4 (30.8)	6 (46.2)	0.4201	7 (53.8)	3 (23.1)	0.1068	9 (69.2)	1 (7.7)	0.0012*
	Fusiform/Epithelioid	9 (69.2)	7 (53.8)		6 (46.2)	10 (76.9)		4 (30.8)	12 (92.3)	
AJCC-stage	III	5 (38.5)	4 (30.8)	0.2110	5 (38.5)	4 (30.8)	0.6969	5 (38.4)	4 (30.8)	0.8948
	IVa	6 (46.2)	3 (23.1)		5 (38.4)	4 (30.8)		4 (30.8)	5 (38.4)	
	IVb and IVc	2 (15.3)	6 (46.1)		3 (23.1)	5 (38.5)		4 (30.8)	4 (30.8)	

AMT, mitochondrial antibody; FIS1, fission 1 protein; MFN2, mitofusin 2; AJCC, American Joint Committee on Cancer; CH, chemotherapy; RT, radiotherapy.
Asterisks (*) indicate statistical significance (P<0.05).

Table 6. Relationship of clinicopathological characteristics of patients with sinonasal melanomas (n=26), AMT, FIS1, and MFN2 expressions with OS rates in univariate and multivariate models.

Variables	**Categories**		OS univariate (%)		P-value (log-rank)	Multivariate	
		n (%)	3-years	5-years		HR (95% CI)	P-value
Age	<59	13 (50.0)	59.2	39.5	0.67	–	–
	≥59	13 (50.0)	53.9	40.5	(0.17)		
Sex	Female	14 (53.8)	66.2	41.4	0.53	–	–
	Male	12 (46.2)	48.6	36.5	(0.38)		
Anatomical site	Nasal cavity	11 (42.3)	57.7	57.7	0.32	–	–
	Maxillary sinus	10 (38.5)	54.9	54.9	(2.24)		
	Rinopharynx	5 (19.2)	60.0	0.0			
Treatment	Only surgery	11 (42.3)	57.1	28.6	0.24	–	–
	Surgery plus CH/RT	14 (53.8)	60.6	50.5	(1.36)		
	No treatment	1 (3.9)	–	–			
Vascular invasion	Absent	19 (73.1)	70.1	50.1	0.01*	2.16	0.15
	Present	7 (26.9)	28.6	14.3	(5.60)	(0.75 – 6.19)	
Neural invasion	Absent	24 (92.3)	63.0	43.0	0.12	–	–
	Present	2 (7.7)	0.0	0.0	(2.33)		
Mitotic index (mm^2)	<1	18 (69.2)	55.8	55.8	0.79	–	–
	≥1	8 (30.8)	62.5	15.6	(0.06)		
Necrosis	Absent	16 (61.5)	61.3	42.0	0.27	–	–
	Present	10 (38.5)	50.0	33.3	(1.21)		
Cellular morphology	Plasmacytoid/others	10 (38.5)	100.0	83.3	<0.01*	12.840	0.02*
	Epithelioid/Fusiform	16 (61.5)	37.5	22.5	(10.335)	(1.30 –126.37)	
AJCC–stage	III	9 (34.6)	85.7	64.3	0.21	–	–
	IVa	9 (34.6)	43.8	29.2	(3.12)		
	IVb and IVc	8 (30.8)	37.5	18.8			
AMT	Low	13 (50.0)	33.8	33.8	0.07	–	–
	High	13 (50.0)	76.2	48.4	(3.14)		
FIS1	Low	13 (50.0)	80.0	53.3	0.057	–	–
	High	13 (50.0)	35.9	23.9	(3.63)		
MFN2	Low	13 (50.0)	80.2	50.1	0.02*	0.88	0.85
	High	13 (50.0)	38.5	28.8	(5.21)	(0.23 – 3.25)	

AMT, mitochondrial antibody; FIS1, fission 1 protein; MFN2, mitofusin 2; AJCC, American Joint Committee on Cancer; OS, overall survival, HR, Harzard Ratio; CI, Confidence interval.

Asterisks (*) indicate statistical significance (P<0.05).

Univariate Cox analysis of all cutaneous cases showed a significant correlation between lower disease-free survival (DFS) rates and presence of ulceration, Breslow's thickness, Clark's level, mitotic index, AJCC-stage, AMT, FIS1 and MFN2 expressions. While, in the multivariate Cox analysis of all cutaneous cases (including all variables with significance in the univariate model) only Breslow's thickness, mitotic index and AMT expression were significant for DFS. With respect to overall survival (OS), in the univariate model, ulceration, Breslow's thickness, mitotic index, AJCC-stage, AMT and MFN2 expressions demonstrated significant prognostic value. All variables, except AJCC-stage, lost the prognostic value within a multivariate model (summarized in Table 2).

Moreover, the predictive value of tumor ulceration status, Breslow's thickness, mitotic rate, Clark's level, and the expression of AMT, FIS1 and MFN2 expressions for distant metastasis risk was assessed by Cox analysis. Only presence of ulceration (HR: 4.29, 95% CI: 1.19 – 15.37, *P*=0.025) and MFN2 expression (HR: 38.88, 95% CI: 3.42 – 441.93, *P*=0.003) were significantly correlated with high risk for distant metastasis, in a multivariate model (Supplementary Table 2).

Oral melanomas

Thirty patients with oral melanomas (16 female, 14 male) were included. Regarding the site distribution 13 cases occurred in the palate, 5 in the inferior gingiva and 12 cases in other localizations that include the tongue (1 case) and floor of the mouth (1 case), six cases have no information about the specific site. The distribution of AJCC-stages was as follows: III 43.3% (13/30); IVa 36.7% (11/30); IVb and IVc 20% (6/30).

High AMT expression was associated with epithelioid cellular morphology, whereas high FIS1 expression was correlated with the presence of vascular invasion,

mitotic index and AJCC-stage. MFN2 expression was significantly associated only with AJCC-stage, with most cases with IVb/IVc stages presenting low expression of this marker (Table 3). Regarding the OS probabilities, some clinicopathologic characteristics were significantly correlated with lower OS, including anatomical site (palate), the presence of vascular and neural invasion, high mitotic index, epithelioid cellular morphology, advanced AJCC-stages, AMT and FIS1 expressions. High MFN2 expression demonstrated a better prognosis, without statistical significance. In the multivariate model, anatomical site (palate), AMT and FIS1 were significantly correlated with lower OS rates (summarized in Table 4).

Sinonasal melanomas

Twenty-six patients with oral melanomas (14 female, 12 male) were included. Regarding the site distribution 11 cases occurred in the nasal cavity, 10 in the maxillary sinus and 5 cases in the rinopharynx. The distribution of AJCC-stages was as follows: III 34.6% (9/26), IVa 34.6% (9/26); IVb and IVc 30.8% (8/26).

High expression of FIS1 was significantly correlated with the presence of vascular invasion, whereas MFN2 expression was associated with age (high expression in young patients), sex (high expression in men), and fusiform/epithelioid cellular morphology (Table 5). Lower OS rates were significantly associated with the presence of vascular invasion and fusiform/epithelioid cellular morphology. Whereas, regarding the mitochondrial markers, only MFN2 was associated with lower OS probability. In the multivariate model, fusiform/epithelioid cellular morphology showed predictive value for sinonasal melanomas (summarized in Table 6).

DISCUSSION

There are increasing shreds of evidence that mitochondrial dynamics regulate melanogenesis and probably has an important role in the malignant transformation of melanocytes [18]. Besides that, increased metabolism of tumor cells is a hallmark of cancer progression, and mitochondria are directly linked with energy production in human cells [19]. Corroborating with this, there has been studied a new tool for predicting melanoma metastasis using a magnetic resonance imaging method is based in the mitochondrial redox ratio, and it has an important value for predict and diagnosis of melanoma metastases [20]. In cancer cells, mitochondria constantly modify their number, shape and function mainly through two main processes (fission and fusion) and generically named mitochondrial dynamics [1,4]. The inhibition of mitochondrial dynamics-related proteins is also associated with reduced growth tumor [21]. However, limited research exists on the immunohistochemical expression of the mitochondrial markers and their prognostic value in mucosal and cutaneous melanomas. In the current study, we carried out a retrospective study to assess the expression of three mitochondrial markers in a series of cutaneous and mucosal (oral and sinonasal) melanomas. Furthermore, we evaluated the potential prognostic value of the mitochondrial content (assessed by AMT expression), and two proteins involved in the fission (FIS1) and fusion (MFN2) processes.

One interesting finding of this current study was the high mitochondrial content observed in MM than in CM, indicating that probably the mitochondrial mass may influence in the poor prognosis of MM. In agreement with prior reports high mitochondrial content is associated with poor prognosis in several human cancers such as head and neck squamous cell carcinomas [22], gallbladder [23], prostate [24] and breast carcinomas [25]. In addition, some studies have demonstrated a crucial role of the

mitochondrial mass in acquisition of a malignant phenotype and in tumor chemo-resistance [26,27]. Our findings also demonstrated that high mitochondrial content is an independent prognostic marker for DFS in cutaneous and OS in oral melanomas. In cutaneous melanomas, high expression of AMT was associated with AJCC-stage and Breslow's thickness, indicating a possible role of mitochondrial mass in cutaneous melanoma pathogenesis. High expression of AMT is also correlated with ulceration, indeed in ulcerated lesions; we observed a subpopulation of macrophages strongly positive for AMT. Thus, we hypothesize that the inflammatory infiltrate that overexpress AMT may be associated with the high mitochondrial content. As demonstrated previously, mitochondrial biogenesis plays a crucial role during cell cycle progression [28], and it corroborates with a positive correlation between high expression of AMT and mitotic index in cutaneous melanomas.

With respect to the fission process, several proteins are involved in its signaling, including dynamin-related protein 1 (DRP1) and fission protein 1 (FIS1) [29]. Recently, several studies have addressed the oncogenic role of DRP1 in cellular responses to MAPK inhibition [30]. In addition, another study showed that the induction of DRP1 in nevi and melanoma contributed to BRAFV600E disease [31]. Thus, it was hypothesized that the fission process could drive essential events for melanoma progression. Regarding FIS1 expression, in this study, we demonstrated a correlation between Clark's levels with increased FIS1 immunostaining. In fact, different Clark's levels comprise an interesting example to study cutaneous melanoma progress. Besides that, FIS1 expression was correlated with critical prognostic parameters for cutaneous melanomas, such as Breslow's thickness and mitotic index. In oral melanomas, FIS1 expression was associated with AJCC-stage and strictly correlated with vascular invasion. We have also identified FIS1 as an independent prognostic factor for OS in

patients with oral melanomas in both univariate and multivariate models. Importantly, our data suggest that FIS1 plays an essential role in the pathogenesis of cutaneous and oral melanomas, and consequently has identified as a possible prognostic biomarker for these tumors, mainly oral melanomas.

The exact role of MFN2 in cancer is not well established, with contradictory data, in some studies has demonstrated an oncogenic activity and other studies have studied its antioncogenic effect [13,32]. Concerning MFN2 expression in our cohort of head and neck melanomas, it was associated with lower DFS and OS in the multivariate model for patients with cutaneous melanomas. Furthermore, high MFN2 expression demonstrated a correlation with high risk for distant metastasis in a multivariate model. In oral melanomas, cases with high MFN2 expression have a better prognosis than cases with lower expression. Although this data was not significant, we consider important to highlight a possible antioncogenic effect of MFN2 in these particular tumors. It is in accordance with a previous study, in which, B16F10 melanoma cells knockdown for MFN2 demonstrated a higher number of lung metastasis [32]. On the other hand, for sinonasal melanomas, MFN2 was also associated with worse OS rates in the univariate model, corroborating with other studies [13,15]. Additional studies to determine the role of MFN2 in different human cancers types are highly suggested.

Current understanding of the clinical and pathological characteristics of mucosal melanomas is mainly based on the analysis of individual cases or small series [33–35]. However, in this study, we collected 56 oral and sinonasal melanomas samples with strict follow-up and complete clinicopathologic data. To the clinicopathologic parameters for oral melanomas, we identified a worse outcome for lesions in the palate and demonstrated a prognostic value of AMT and FIS1 for these tumors. In a univariate model, variables as vascular and neural invasion, mitotic index and AJCC-stage were

significantly correlated with lower OS rates. Importantly, epithelioid cellular morphology has emerged as a valuable prognostic tool, corroborating with previous studies. Regarding the sinonasal melanomas, only an epithelioid/fusiform cellular morphology was identified as an independent prognostic factor. Whereas in the univariate model, vascular invasion and MFN2 were correlated with lower OS probability. The lack of predictive value of these clinicopathologic parameters probably is due to the heterogeneity of these tumors, and future studies must be able to study the melanomas of the nasal cavity separately and from other localizations such as paranasal sinuses and rinopharynx.

In conclusion, high expression of AMT and FIS1 are associated with the aggressive characteristics and poor prognosis in oral melanomas. We also demonstrated the predictive value of MFN2 for high metastatic risk in cutaneous melanomas of the head and neck region. The novel mitochondrial markers that we identified may help in the development of new drugs targeting the mitochondria and modulating the mitochondrial dysfunction that underlies the pathogenesis of mucosal and cutaneous melanomas to prevent tumor recurrence and distant metastasis.

CONFLICTS OF INTEREST

The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS

Conception and design: CDS, JJ, OPA

Provision of study materials or patients: FVMBC, MBC, RRDR, LSA, RC, AMMA, MGFC, OPA

Collection and assembly of data: CDS, MGFC, OPA, TMLM

Data analysis and interpretation: CDS, TMLM

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Supplementary Tables

Supplementary Table 1. List of primary antibodies, clone, dilutions, source and positive controls used in this study.

Antibody	Clone	Dilution	Source	Positive control
Anti-mitochondrial	113-1	1:100	BioGenex[a]	Liver
FIS1	B-5	1:50	Santa Cruz Biotechnology[b]	Oncocytoma
MFN2	XX-1	1:50	Santa Cruz Biotechnology[b]	Warthin's tumor

[a]Fremont, CA, USA; [b]Dallas, TX, USA.

Supplementary Table 2. Multivariate analysis of risk factors for metastasis of head and neck cutaneous melanomas based on clinicopathological characteristics and AMT, FIS1 and MFN2 expressions.

Factors	*P*-value	HR	95% CI	
			Superior	Inferior
Ulceration	0.025*	4.292	1.198	15.374
Breslow's thickness	0.388	1.777	0.482	6.545
Mitotic index	0.439	0.620	0.185	2.081
Clark's level	0.776	1.522	0.084	27.614
AMT	0.112	3.856	0.730	20.359
FIS1	0.262	0.149	0.005	4.146
MFN2	0.003*	38.883	3.421	441.930

HR, Hazard ratio; CI, confidence interval; AMT, mitochondrial antibody; FIS1, fission 1 protein; MFN2, mitofusin 2.
Asterisks (*) indicate statistical significance (P<0.05).

3 DISCUSSÃO

Visto que, em cada seção específica do artigo, os aspectos mais relevantes já foram discutidos, nesse espaço apenas ressaltaremos alguns resultados que consideramos importantes de cada capítulo e apresentaremos uma visão geral das principais conclusões desse trabalho.

Melanomas cutâneos e mucosos são neoplasias totalmente distintas do ponto de vista biológico. De fato, esses tumores são bastante agressivos e apresentam uma grande tendência a metástases (Braeuer *et al.*, 2014). Nesse estudo, nosso principal objetivo foi avaliar o papel biológico e o valor prognóstico de p-Akt1, COX-2 e marcadores mitocondriais nestas neoplasias. O primeiro capítulo deste estudo consistiu em avaliar o papel prognóstico da proteína p-Akt1 em melanomas cutâneos e mucosos. Estudos anteriores já haviam demonstrado que o AKT1 está associado a um fenótipo de melanoma mais metastático em modelo experimental (Cho *et al.*, 2015), no entanto, nós demonstramos pela primeira vez, que os melanomas mucosos (orais e nasais) apresentam maior expressão de p-Akt1 do que os melanomas cutâneos. Além disso, em conjunto com os dados demonstrados por estudos anteriores e, o fato de p-Akt1 ser um fator prognóstico independente para os melanomas mucosos pode indicar que novas terapias-alvo sejam desenvolvidas a partir desses resultados.

O segundo capítulo trata de um estudo determinou o papel da enzima COX-2 como um marcador prognóstico imuno-histoquímico importante para melanoma oral. Assim, estudos adicionais que estudem as diferentes vias de sinalização que ativam COX-2 em melanoma oral são necessários, para validar essa enzima como um potencial alvo terapêutico em melanomas mucosos.

No capítulo 3 e 4, apresentamos uma série de casos de melanomas orais amelanóticos e comparamos a expressão de COX-2 e de Ki67 nesses tumores e em melanomas orais convencionais, com deposição de melanina. Os melanomas amelanóticos parecem ter maior agressividade tumoral. Tanto os aspectos clínicos, como os aspectos patológicos confirmam essa hipótese. Os melanomas amelanóticos cutâneos também são considerados mais agressivos que os convencionais e vários fatores tem sido atribuídos a maior agressividade tumoral, incluindo que estes tumores podem representar uma variante pouco diferenciada e portanto, maior tendência a metástase e pior prognóstico (Thomas *et al.*, 2014; Moshe *et al.*, 2018).

No capítulo 5, foi estudada a expressão de marcadores mitocondriais em melanomas cutâneos e mucosos. Dentre eles, a proteína anti-mitocondrial (AMT), proteína de fissão 1 (FIS1) e proteína de fusão 2 (MFN2). A quantidade de mitocôndria, avaliada subjetivamente pela intensidade de AMT nas células tumorais parece desempenhar um papel importante na patogênese de melanomas; particularmente, os melanomas mucosos que apresentaram uma maior expressão de AMT. A proteína FIS1 teve maior expressão em melanomais orais e interessantemente pode ser considerada um fator prognóstico independente nesses tumores. Por outro lado, os melanomas nasais apresentaram alta expressão de MFN2 em comparação com os melanomas orais e cutâneos e também demonstrou relação com pior prognóstico em pacientes com melanomas cutâneos (Soares *et al.*, 2018).

Confirmamos em todos os capítulos da presente tese que os melanomas de mucosa oral e nasal tem um comportamento biológico distinto dos melanomas cutâneos. De fato, os melanomas mucosos apresentam uma expressão aumentada de p-Akt1, COX-2 e de marcadores mitocondriais comparado aos melanomas cutâneos. Esperamos que esses resultados, em conjunto, possam auxiliar no desenvolvimento de novas terapias-alvo objetivando ampliar o arsenal de opções terapêuticas dessas neoplasias malignas extremamente agressivas e com prognóstico sombrio.

4 CONCLUSÃO

O presente estudo confirmou que os melanomas de mucosa oral e nasal tem uma biologia muito distinta dos melanomas cutâneos convencionais. Características clínicas e patológicas como idade, tamanho do tumor, tipo celular e estadiamento podem influenciar negativamente a sobrevida dos pacientes. De maneira mais relevante, demonstramos que p-Akt1 e COX-2 podem ser usados como marcadores prognósticos em melanomas mucosos, e consequentemente podem desempenhar papel importante na biologia tumoral e no desenvolvimento de terapias-alvo.

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*De acordo com as normas da UNICAMP/FOP, baseadas na padronização do International Committee of Medical Journal Editors - Vancouver Group. Abreviatura dos periódicos em conformidade com o PubMed.

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ANEXOS

ANEXO 1 - VERIFICAÇÃO DE ORIGINALIDADE E PREVENÇÃO DE PLÁGIO

ESTUDO COMPARATIVO DA EXPRESSÃO DE p-AKT1, COX-2 E MARCADORES MITOCONDRIAIS EM MELANOMAS CUTÂNEOS E MUCOSOS

ORIGINALITY REPORT

11%	5%	11%	3%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	Ciro Dantas Soares, Thayná Melo de Lima Morais, Roman Carlos, Oslei Paes de Almeida et al. "Prognostic importance of mitochondrial markers in mucosal and cutaneous head and neck melanomas", Human Pathology, 2018 Publication	2%
2	de Souza do Nascimento, Juliana, Román Carlos, Wilson Delgado-Azañero, Adalberto Mosqueda Taylor, Oslei Paes de Almeida, Mário José Romañach, and Bruno Augusto Benevenuto de Andrade. "Immunohistochemical expression of cyclooxygenase-2 (COX-2) in oral nevi and melanoma", Journal of Oral Pathology and Medicine, 2015. Publication	2%
3	Michael Moshe, Assi Levi, Dean Ad-El, Dan Ben-Amitai, Daniel Mimouni, Elena Didkovsky, Meora Feinmesser, Moshe Lapidoth. "Malignant melanoma clinically mimicking pyogenic	1%

ANEXO 2 – PARECER CONSUBSTANCIADO DA COMISSÃO NACIONAL DE ÉTICA EM PESQUISA (CONEP)

COMISSÃO NACIONAL DE
ÉTICA EM PESQUISA

PARECER CONSUBSTANCIADO DA CONEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: PROJETO INVESTIGATIVO PARA CARACTERIZAR O PERFIL MICROBIÔMICO E A RESPOSTA IMUNOLÓGICA EM MELANOMAS PRIMÁRIOS E METASTÁTICOS DE PELE, CAVIDADE ORAL, E NASOFARINGE

Pesquisador: CIRO DANTAS SOARES

Área Temática: A critério do CEP

Versão: 5

CAAE: 72077517.1.0000.5418

Instituição Proponente: Faculdade de Odontologia de Piracicaba - Unicamp

Patrocinador Principal: FUNDACAO DE AMPARO A PESQUISA DO ESTADO DE SAO PAULO

DADOS DO PARECER

Número do Parecer: 2.524.225

Situação do Parecer:
Aprovado

ANEXO 3 – PÁGINA INICIAL DO ARTIGO 2.1

www.oncotarget.com Oncotarget, 2018, Vol. 9, (No. 99), pp: 37291-37304

Research Paper

Phosphorylated Akt1 expression is associated with poor prognosis in cutaneous, oral and sinonasal melanomas

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Keywords: *cutaneous melanomas; mucosal melanomas; p-Akt1; immunohistochemistry; prognosis*

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ABSTRACT

Melanomas are highly aggressive tumours derived from melanocytes, which occur most commonly in the skin. Occasionally, these tumours may appear in oral and sinonasal mucous membranes. In this study, we performed a comparative analysis of the Phosphorylated Akt1 (p-Akt1) expression in 144 patients affected by cutaneous (CM), 34 oral cavity (OM), and 31 sinonasal melanomas (SNM). Similar to the metastatic cutaneous melanomas, p-Akt1 was overexpressed in 17/34 of the oral cavity and 20/31 of the sinonasal melanomas. In addition, the p-Akt1-nuclear expression was associated with poorer cancer-specific survival in cutaneous ($P < .0001$), oral ($P < .0001$), and sinonasal ($P = .001$) melanomas. Multivariate analysis showed p-Akt1 to be an independent prognostic marker in oral ($P = .041$) and sinonasal ($P < .0001$) melanomas patients. In conclusion, p-Akt1 overexpression is an independent prognostic marker in mucosal melanomas and is significantly up-regulated in sinonasal melanomas. As both mucosal and metastatic cutaneous melanomas showed high frequency of p-Akt1 expression, these findings suggest that mucosal melanomas have a biological behaviour, similar to the aggressive cutaneous melanomas.

INTRODUCTION

Cutaneous melanomas represent about 1.6% of all cancers, and discreet advances in their treatment have been made over the last decades. Nevertheless, this tumour still remains deathly, especially the metastatic disease [1]. It is estimated that melanomas with lymph nodal metastases are responsible for 59,782 global deaths, with mean overall survival rate of 13% in 5 years [2]. Controlling the advanced-stage disease is the major problem for the treatment of melanomas. Thus, for the development of targeted therapies, making progress in understanding the molecular factors that influence the aggressiveness of the metastatic melanomas is essential.

ANEXO 4 – PÁGINA INICIAL DO ARTIGO 2.5

ELSEVIER

Human Pathology
Volume 85, March 2019, Pages 279-289

Original contribution

Prognostic importance of mitochondrial markers in mucosal and cutaneous head and neck melanomas ☆ ☆☆

Ciro Dantas Soares DDs, MSc, PhD student [a], Thayná Melo de Lima Morais DDs, MSc, PhD student [a], Roman Carlos DDs [b], Oslei Paes de Almeida DDs, MSc, PhD [a], Fernanda Viviane Mariano DDs, MSc, PhD [a, c], Albina Altemani MD, PhD [a, c], Maria Goretti Freire de Carvalho MD, PhD [d], Marcelo Brum Corrêa MD [e], Rodrigo Ribas Dias dos Reis MD [f], Luciana Schultz Amorim MD, PhD [g], Jacks Jorge DDs, MSc, PhD [a]

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https://doi.org/10.1016/j.humpath.2018.11.009

Highlights

- High content of mitochondria may play an important role in melanoma pathogenesis.
- FIS1 expression predicts lower overall survival rates in oral melanoma patients.
- FIS1 expression is associated with vascular invasion in mucosal melanoma patients.
- MFN2 expression predicts metastasis in cutaneous melanoma patients.