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Original contribution

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

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Summary Mitochondrial dysfunction is caused by an imbalance in the fission and fusion processes, and it has been implicated in the pathogenesis of several human cancers. However, the role of mitochondrial markers in melanomas still remains poorly understood. In this study, the authors assessed the expression of 3 mitochondrial markers (antimitochondrial, fission protein 1 [FIS1], and mitofusin 2 [MFN2]) in a series of head and neck mucosal and cutaneous melanomas. Patients with cutaneous (n = 56) and mucosal (oral, n = 30, sinonasal, n = 26) melanomas of the head and neck region were enrolled in this study. Clinical and follow-up data were retrieved from medical records. The expression of 3 mitochondrial markers was assessed by the immunohistochemistry, and then 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 in oral melanomas. FIS1 expression was significantly associated with lower overall survival rates in patients with oral melanomas and strictly correlated with vascular invasion in mucosal melanomas. MFN2 was

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associated with high risk of distant metastasis in patients with cutaneous melanomas. In summary, the authors demonstrated that mitochondrial content, along with FIS1 and MFN2 expressions, is correlated with important clinicopathological characteristics in patients with cutaneous and mucosal head and neck melanomas.

1. Introduction

Mitochondria are highly dynamic organelles involved in the energy production of all human cells [1], and oxidative phosphorylation is the main source of energy for several biological processes that demand ATP [2]. As a consequence of oxidative phosphorylation, mitochondria also produce most reactive oxygen species (ROS). ROS are implicated in the carcinogenic process, damaging important proteins and macromolecules, causing cell cycle deregulation and other relevant processes that lead to 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, the predictive value of mitochondrial markers is described in recurrence, metastasis, and tamoxifen-resistance of breast cancer patients [5]. In the intracellular compartment, mitochondria constantly change in number and shape in an event termed *mitochondrial dynamics*, which involves 2 main processes: fusion and fission [1]. Although such processes are widely investigated, their role in cancer cells and consequent clinical behavior, tumorigenesis, and metastasis remains poorly studied in the context of melanomas.

Melanomas are aggressive tumors that arise most commonly on the skin or mucous membranes. Prognosis of primary melanoma is based on morphologic parameters such as the tumor thickness and the mitotic index [6,7]. Considering that cutaneous melanomas (CM) are strictly associated with sun exposure, whereas in mucosal melanomas (MMs), such association has no effect in their pathogenesis, comparative studies are essential to clarify the differences in signaling pathways involved in the different melanomas subtypes [8,9]. In addition, it has been currently demonstrated that CM and MM harbor different genetic alterations, and few studies have compared mucosal and cutaneous head and neck (H&N) melanomas [10]. Oral and sinonasal melanomas are uncommon tumors that arise in the mucosa of these anatomical sites, presenting aggressive behavior, tendency to metastasize, and worse prognosis than CMs [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 established [13-15]. Furthermore, high ROS levels are associated with tumor development and progression, and as the main sources of ROS are mitochondria, it is hypothesized that these organelles may play an essential role in tumorigenesis and

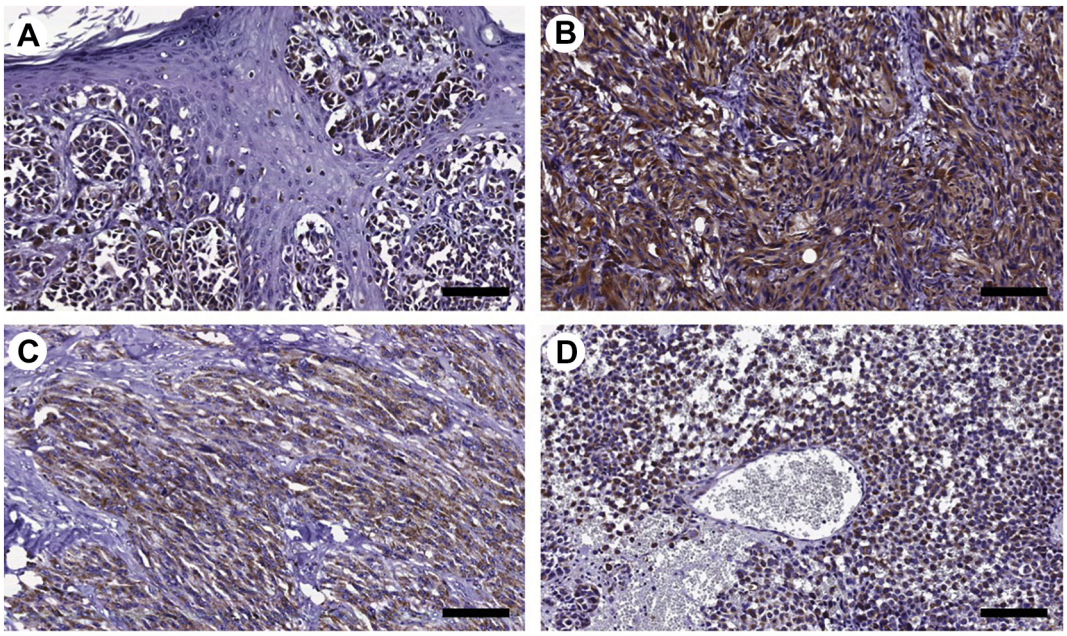

Fig. 1 AMT expression in cutaneous and mucosal H&N melanomas. A, Medium cytoplasmic positivity in malignant melanocytes in CM. B, Strong positivity in a predominant fusiform oral melanoma. C, Strong positivity in a spindle/epithelioid sinonasal melanoma. D, Perinuclear pattern of AMT immunostaining in sinonasal melanomas, undifferentiated type. Scale bars represent 100 μm for all figures.

impacts in the biological behavior of several cancers [16]. However, in melanomas, this role is not fully clarified. In the current study, the expression of 3 mitochondrial markers in oral, sinonasal, and cutaneous of the H&N region melanomas was compared for their further comprehension.

2. Materials and methods

2.1. Case selection

A total of 112 H&N cutaneous and MM cases with follow-up and complete clinical information from 4 pathology laboratories were retrospectively collected. Formalin-fixed, paraffin-embedded tissue blocks were retrieved; the melanomas diagnoses were reviewed and confirmed by 3 pathologists. In addition, S-100, MelanA, and HMB45 immunostainings were performed in all cases.

2.2. Immunohistochemistry

For the 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. After that, endogenous peroxidase activity was blocked with 20% H_2O_2 with a single 15-minute incubation.

The sections were then incubated with the diluted primary antibodies for 2 hours at room temperature. Two high-sensitive visualization systems were used: ADVANCE/HRP (code K406889-2; Dako, Carpinteria, CA) and EnVision G|2 System/AP, Rabbit/Mouse (Permanent Red; code K535521-2; Dako). The IHQ reactions were revealed with 3,3′-diaminobenzidine (Sigma-Aldrich, St Louis, MO) or Permanent Red (Dako), and counterstained with Carazzi hematoxylin. In cases stained with 3,3′-diaminobenzidine, bleaching was performed following the protocol previously established by our group [17]. Detailed

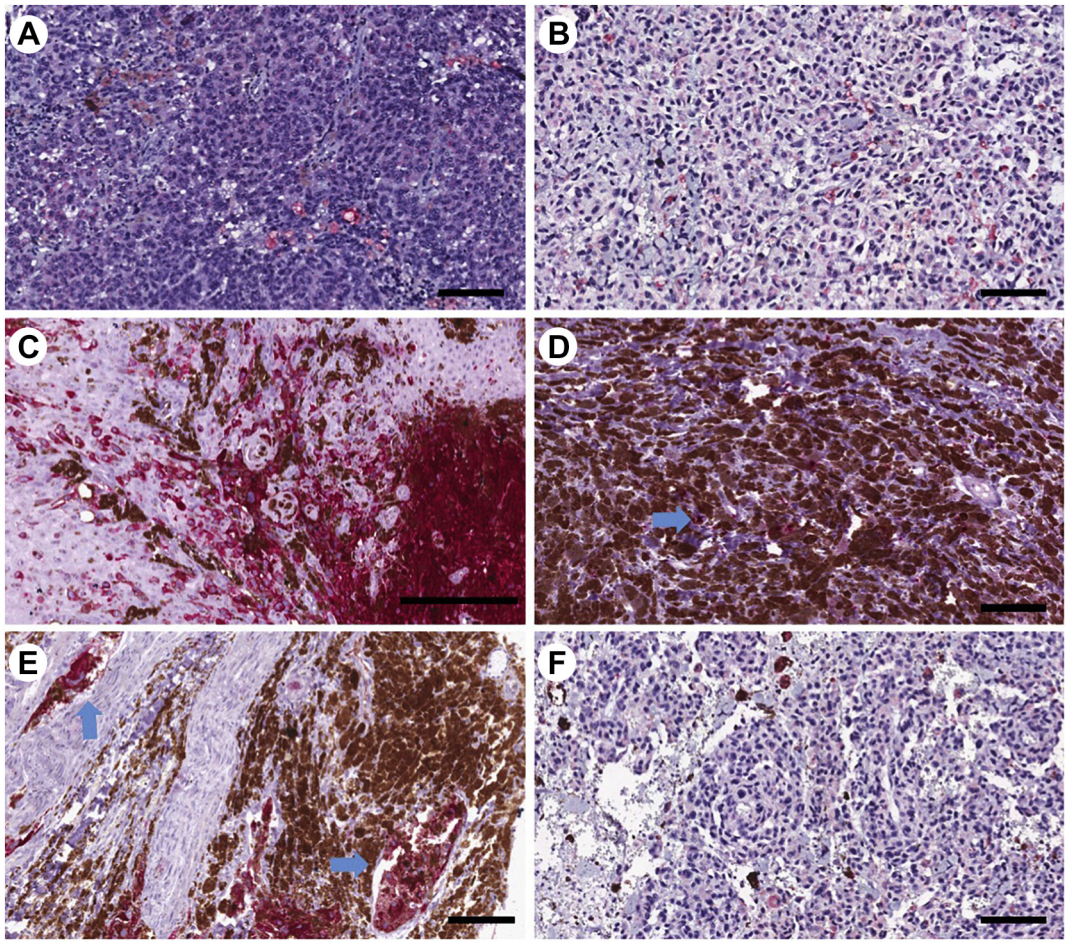

Fig. 2 FIS1 expression in cutaneous and mucosal H&N melanomas. A, Medium cytoplasmic positivity in macrophages and very weak FIS1 expression in CM. B, Weak cytoplasmic expression in tumor cells of CM. C, Strong FIS1 expression in malignant melanocytes of oral melanoma. D, FIS1-positive cells during mitosis (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. Scale bars represent 100-μm magnification for all figures, except for panel C (200 μm).

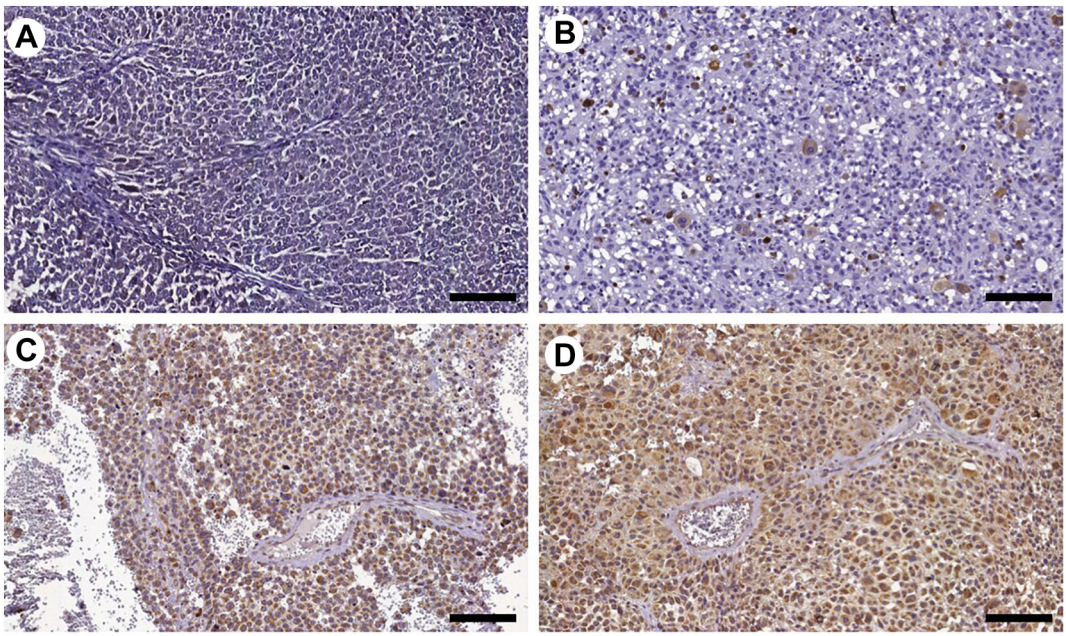

Fig. 3 MFN2 expression in cutaneous and mucosal H&N melanomas. A, Medium MFN2 immunostaining in CM. B, MFN2 positivity was focal and in individual cells of oral melanomas. C, Perinuclear MFN2 immunopositivity was observed in some cases of sinonasal melanomas. D, Strong MFN2 positivity in a case of sinonasal melanoma (stage IVc). Scale bars represent 100 μm for all figures.

information on the primary antibodies, dilution, manufacturer, and respective positive controls is listed in Supplementary Table 1. Negative controls were obtained by omission of the primary antibodies.

2.3. Digital analysis

After the IHC reactions, the slides were scanned into high-resolution images and digitally assessed as described previously with the scores of positivity ranging from 100 (very weak) to 300 (strongly positive) [17]. One calibrated pathologist selected 10 different and randomized regions per case to perform the digital IHC quantification; these areas were from the superior and inferior portions of the tumors. In cases stained with permanent red, the software distinguishes red (IHC signal) from brown (melanin) areas; nevertheless, highly pigmented areas were avoided.

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

Variables	Categories	AMT, n (%)		*P*	FIS1, n (%)		*P*	MFN2, n (%)		*P*
		Low	High		Low	High		Low	High	
Age	<56 y	14 (51.9)	15 (51.7)	.9923	14 (50.0)	15 (53.6)	.7891	13 (46.4)	16 (57.1)	.4223
	≥56 y	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)	.6106	13 (46.4)	12 (42.9)	.7880	13 (46.4)	12 (42.9)	.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)	.0116 *	24 (85.7)	10 (35.7)	.0001 *	24 (85.7)	10 (35.7)	.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)	.7395	15 (53.6)	7 (25.0)	.0286 *	13 (46.4)	9 (32.1)	.2737
	Vertical	17 (63.0)	17 (58.6)		13 (47.4)	21 (75.0)		15 (53.6)	19 (67.9)	
Breslow thickness	<3.3 mm	23 (85.2)	16 (55.2)	.0146 *	25 (89.3)	14 (50.0)	.0013 *	24 (85.7)	15 (53.6)	.0089 *
	≥3.3 mm	4 (14.8)	13 (44.8)		3 (10.7)	14 (50.0)		4 (14.3)	13 (46.4)	
Clark level	I, II, III	15 (55.6)	10 (34.5)	.1129	22 (78.6)	3 (10.7)	<.0001 *	17 (60.7)	8 (28.6)	.0155 *
	IV, V	12 (44.4)	19 (65.5)		6 (21.4)	25 (89.3)		11 (39.3)	20 (71.4)	
Mitotic index	<3 mm^2	16 (59.3)	8 (27.6)	.0167 *	19 (67.9)	5 (17.9)	.0001 *	19 (67.9)	5 (17.9)	.0001 *
	≥3 mm^2	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)	<.0001 *	19 (67.9)	14 (50.0)	.1744	24 (85.7)	9 (32.1)	<.0001 *
	III and IV	3 (15.0)	17 (85.0)		9 (45.0)	11 (55.0)		4 (20.0)	16 (80.0)	

* Statistical significance ($P < .05$).

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

Variables	Categories	n (%)	DFS univariate (%)		*P* (log-rank)	Multivariate		OS univariate (%)		*P* (log-rank)	Multivariate	
			3 y	5 y		HR (95% CI)	*P*	3 y	5 y		HR (95% CI)	*P*
Age	<56 y	29 (51.8)	93.1	77.3	.29	–	–	93.1	81.6	.21	–	–
	≥56 y	27 (48.2)	92.3	59.9	(1.09)			92.3	67.6	(1.57)		
Sex	Female	25 (44.6)	95.7	77.0	.39	–	–	95.7	82.2	.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	<.01 *	3.27	.08	97.1	90.9	<.01 *	2.23	.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	.47	–	–	95.5	76.4	.24	–	–
	Vertical	34 (60.7)	87.7	67.5	(0.51)			87.8	74.4	(1.34)		
Breslow thickness	<3.3 mm	39 (69.5)	97.4	86.4	<.01 *	4.61	.02 *	97.4	86.4	<.01 *	4.593	.052
	≥3.3 mm	17 (30.5)	81.6	30.1	(13.43)	(1.24-17.11)		81.6	48.3	(7.69)	(0.98-21.43)	
Clark level	I, II, III	25 (44.6)	95.8	77.9	.02 *	4.32	.44	100.0	82.8	.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 mm^2	24 (42.8)	95.8	91.7	<.01 *	5.168	.04 *	95.8	91.7	<.01 *	2.87	.20
	≥3 mm^2	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 (62.3)	96.6	85.5	<.01 *	2.469	.18	100.0	96.4	<.01 *	11.21	.03 *
	III and IV	20 (37.7)	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	<.01 *	24.07	.012 *	100.0	95.5	<.01 *	1.32	.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	<.01 *	0.04	.14	96.4	85.6	.06	0.24	.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	<.01 *	1.14	.87	100.0	92.7	<.01 *	2.09	.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)	

* Statistical significance ($P < .05$).

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

Variables	Categories	AMT, n (%)		*P*	FIS1, n (%)		*P*	MFN2, n (%)		*P*
		Low	High		Low	High		Low	High	
Age	<48 y	6 (40.0)	10 (66.7)	.1432	7 (46.7)	9 (60.0)	.4642	7 (46.7)	9 (60.0)	.4642
	≥48 y	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)	.4642	6 (40.0)	10 (66.7)	.1432	10 (66.7)	6 (40.0)	.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)	.8706	5 (33.3)	8 (53.3)	.0641	8 (53.3)	5 (33.3)	.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)	.2465	12 (80.0)	8 (53.3)	.2465	9 (60.0)	11 (73.3)	.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)	.7047	15 (100)	4 (26.7)	<.001 *	9 (60.0)	10 (66.7)	.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)	.3613	14 (93.3)	10 (66.7)	.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)	.0654	11 (73.3)	2 (13.3)	<.001 *	6 (40.0)	7 (46.7)	.7125
	≥1	6 (40.0)	11 (73.3)		4 (26.7)	13 (86.7)		9 (60.0)	8 (53.3)	
Cellular morphology	Nonepithelioid	8 (53.3)	2 (13.3)	.0201 *	7 (46.7)	3 (20.0)	.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)	.4843	8 (53.3)	5 (33.3)	.0233 *	8 (53.3)	5 (33.3)	.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)	

Abbreviations: CH, chemotherapy; RT, radiotherapy.

* Statistical significance ($P < .05$).

2.4. Statistical methods

Briefly, for the statistical methods, the CMs were divided into 2 groups based on the Clark level: (1) I, II, and III ;, and (2) IV and V. The immunostaining scores were correlated with clinical data from all types of melanomas using contingency tables and χ^2 or Fisher exact test. Survival curves were calculated according to the Kaplan-Meier method. The log-rank test was applied for patients with low and high marker expression (cutoff 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, Chicago, IL) with a 95% confidence level ($P \leq .05$).

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

3. Results

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

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	n (%)	OS (%) univariate		*P* (log-rank)	Multivariate	
			3 y	5 y		HR (95% CI)	*P*
Age	<48 y	16 (53.3)	56.3	40.2	.76	–	–
	≥48 y	14 (46.7)	43.8	43.8	(0.09)		
Sex	Female	16 (53.3)	38.4	38.4	.33	–	–
	Male	14 (46.7)	64.3	42.9	(0.94)		
Anatomical site	Palate	13 (43.3)	27.7	0.0	.02 *	0.33	.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	.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	<.01 *	1.61	.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	.03 *	0.82	.81
	Present	6 (20.0)	16.7	16.7	(4.48)	(0.17-3.93)	
Mitotic index	<1 mm^2	13 (43.3)	91.7	91.7	<.01 *	6.75	.18
	≥1 mm^2	17 (56.7)	23.5	11.7	(13.93)	(0.42-109.16)	
Necrosis	Absent	22 (73.3)	51.3	41.0	.59	–	–
	Present	8 (26.7)	50.0	25.0	(0.28)		
Cellular morphology	Nonepithelioid	10 (33.3)	88.9	88.9	<.01 *	8.56	.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	<.01 *	1.25	.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	.03 *	12.97	.01 *
	High	15 (50.0)	40.0	13.3	(4.48)	(1.73-97.09)	
FIS1	Low	15 (50.0)	92.9	63.7	<.01 *	22.99	.03 *
	High	15 (50.0)	13.3	13.3	(15.45)	(1.32-401.12)	
MFN2	Low	15 (50.0)	52.5	28.0	.49	–	–
	High	15 (50.0)	50.3	50.3	(0.47)		

Abbreviations: CH, chemotherapy; RT, radiotherapy.
* Statistical significance ($P < .05$).

3.1. Cutaneous melanomas

A total of 56 patients with H&N CMs (25 female, 31 male) were included. The case distribution of American Joint Committee on Cancer (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 thickness was 3.0 mm (ranging from 0 to 38 mm).

All mitochondrial markers analyzed in this study demonstrated correlation with the presence of ulceration, Breslow thickness, and mitotic index. AMT and FIS1 were associated with AJCC stage, whereas FIS1 and MFN2 were correlated with Clark level. High expression of FIS1 was also correlated with the vertical growth phase (Table 1).

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

In addition, the predictive value of tumor ulceration status, Breslow thickness, mitotic rate, Clark level, and the expressions of AMT, FIS1, and MFN2 for distant metastasis risk were assessed with Cox analysis. Only presence of ulceration (hazard ratio [HR], 4.29; 95% confidence interval [CI], 1.19-15.37; $P = .025$) and MFN2 expression (HR, 38.88; 95% CI, 3.42-441.93; $P = .003$) were significantly associated with high risk for distant metastasis, in a multivariate model (Supplementary Table 2).

3.2. Oral melanomas

Thirty patients with oral melanomas (16 female, 14 male) were considered for this study. Regarding site distribution, 13 cases occurred in the palate, 5 in the inferior gingiva, and 12 in other areas that included the tongue (1 case) the mouth floor (1 case), and 6 cases had no specification regarding their incidence areas. The distribution of AJCC stages was as follows: III, 43.3% (13/30); IVa, 36.7% (11/30); and 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 indexing, and AJCC stage. MFN2 expression was significantly

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

Variables	Categories	AMT, n (%)		*P*	FIS1, n (%)		*P*	MFN2, n (%)		*P*
		Low	High		Low	High		Low	High	
Age	<59 y	6 (46.2)	7 (53.8)	.6948	7 (53.8)	6 (46.2)	.6948	4 (30.8)	9 (69.2)	.0498 *
	≥59 y	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)	.4314	8 (61.5)	6 (46.2)	.4314	10 (76.9)	4 (30.8)	.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)	.3180	7 (53.8)	4 (30.8)	.4920	4 (30.8)	7 (53.8)	.4920
	Paranasal sinuses	6 (46.2)	4 (30.8)		4 (30.8)	6 (46.2)		6 (46.1)	4 (30.8)	
	Nasopharynx	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)	.5024	7 (53.8)	4 (30.8)	.3492	5 (38.5)	6 (46.2)	.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)	.6583	12 (92.3)	7 (53.8)	.0270 *	11 (84.7)	8 (61.5)	.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)	.1410	13 (100)	11 (84.7)	.1410	13 (100)	11 (84.7)	.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)	.3954	10 (76.9)	8 (61.5)	.3954	8 (61.5)	10 (76.9)	.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)	.4201	7 (53.8)	3 (23.1)	.1068	9 (69.2)	1 (7.7)	.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)	.2110	5 (38.5)	4 (30.8)	.6969	5 (38.4)	4 (30.8)	.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)	

Abbreviations: CH, chemotherapy; RT, radiotherapy.

* Statistical significance ($P < .05$).

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	n (%)	OS (%) univariate		*P* (log-rank)	Multivariate	
			3 y	5 y		HR (95% CI)	*P*
Age	<59 y	13 (50.0)	59.2	39.5	.67	–	–
	≥59 y	13 (50.0)	53.9	40.5	(0.17)		
Sex	Female	14 (53.8)	66.2	41.4	.53	–	–
	Male	12 (46.2)	48.6	36.5	(0.38)		
Anatomical site	Nasal cavity	11 (42.3)	57.7	57.7	.32	–	–
	Maxillary sinus	10 (38.5)	54.9	54.9	(2.24)		
	Nasopharynx	5 (19.2)	60.0	0.0			
Treatment	Only surgery	11 (42.3)	57.1	28.6	.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	.01 *	2.16	.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	.12	–	–
	Present	2 (7.7)	0.0	0.0	(2.33)		
Mitotic index	<1 mm^2	18 (69.2)	55.8	55.8	.79	–	–
	≥1 mm^2	8 (30.8)	62.5	15.6	(0.06)		
Necrosis	Absent	16 (61.5)	61.3	42.0	.27	–	–
	Present	10 (38.5)	50.0	33.3	(1.21)		
Cellular morphology	Plasmacytoid/others	10 (38.5)	100.0	83.3	<.01 *	12.840	.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	.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	.07	–	–
	High	13 (50.0)	76.2	48.4	(3.14)		
FIS1	Low	13 (50.0)	80.0	53.3	.057	–	–
	High	13 (50.0)	35.9	23.9	(3.63)		
MFN2	Low	13 (50.0)	80.2	50.1	.02 *	0.88	.85
	High	13 (50.0)	38.5	28.8	(5.21)	(0.23-3.25)	

Abbreviations: CH, chemotherapy; RT, radiotherapy.
* Statistical significance ($P < .05$).

associated with AJCC stage only, with most cases within IVb/IVc stages, with low expression of this marker (Table 3). Regarding OS probabilities, some clinicopathological characteristics were significantly associated with lower OS, including the anatomical site (palate), the presence of vascular and neural invasion, high mitotic index, epithelioid cellular morphology, advanced AJCC stages, and AMT and FIS1 expressions. High MFN2 expression was associated with better prognosis, with no statistical significance. In the multivariate model, the anatomical site (palate) and AMT and FIS1 expressions were significantly correlated with lower OS rates (Table 4).

3.3. Sinonasal melanomas

Twenty-six patients with oral melanomas (14 female, 12 male) were considered for this study. Regarding site distribution, 11 cases occurred in the nasal cavity, 10 in the maxillary sinus, and 5 in the nasopharynx area. 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 (higher expression in young patients), sex (high expression within males), and fusiform/epithelioid cellular morphology (Table 5). Lower OS rates were significantly associated with the presence of vascular invasion and fusiform/epithelioid cellular morphology. In contrast, regarding mitochondrial markers, only the MFN2 was associated with lower OS probability. In the multivariate model, fusiform/epithelioid cellular morphology showed predictive value for sinonasal melanomas (Table 6).

4. Discussion

There are increasing shreds of evidence that mitochondrial dynamics regulate melanogenesis and probably have 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 associated with energy production in human cells [19]. Corroborating with this, a new tool for melanoma metastasis prediction has been suggested, which uses a magnetic resonance imaging method based in the mitochondrial redox ratio, and it has been adding important value in the diagnosis and prediction of melanoma metastases [20]. In cancer cells, mitochondria constantly modify their number, shape, and function mainly through 2 main processes (fission and fusion), which are generically termed *mitochondrial dynamics* [1,4]. The inhibition of mitochondrial dynamics–related proteins is also associated with reduced growth tumor [21]. However, limited research on the mitochondrial markers and their prognostic value in mucosal and CMs exists. In this study, a retrospective study was carried out to assess the expression of 3 mitochondrial markers in a series of cutaneous and mucosal (oral and sinonasal) melanomas. Furthermore, the potential prognostic value of the mitochondrial content (assessed by AMT expression) and of 2 proteins involved in the fission (FIS1) and fusion (MFN2) processes were evaluated.

One interesting finding was the higher mitochondrial content observed in MM than in CM, indicating a probability that the mitochondrial mass may influence in the poor MM prognosis. In agreement with prior reports, high mitochondrial content is associated with poor prognosis in several human cancers such as H&N 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 chemoresistance [26,27]. Our findings also demonstrate that high mitochondrial content is an independent prognostic marker for DFS in cutaneous and OS in oral melanomas. In CMs, high expression of AMT was associated with AJCC stage and Breslow thickness, indicating a possible role of mitochondrial mass in CM pathogenesis. High expression of AMT was also ulceration related, as a subpopulation of highly AMT-positive macrophages was observed in ulcerated lesions. Thus, we hypothesize that the inflammatory infiltrate that overexpresses AMT may be associated with the high mitochondrial content. As demonstrated previously, the mitochondrial biogenesis plays a crucial role during cell cycle progression [28], and it corroborates with a positive correlation between high AMT expressions and mitotic index in CMs.

With respect to the fission process, several proteins are involved in its signaling, including the dynamin-related protein 1 (DRP1) and the 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 the development of $BRAF^{V600E}$ disease [31]. Thus, it was hypothesized that the fission process could lead to essential melanoma progression events. Regarding the FIS1 expression, this study demonstrated a correlation between Clark levels with increased FIS1 immunostaining. In fact, different Clark levels are interesting examples to study CM progress. Besides that, the FIS1 expression was correlated with critical prognostic parameters for CMs, such as Breslow thickness and mitotic index. In oral melanomas, the FIS1 expression was associated with the AJCC stage and strictly correlated with vascular invasion. It also identified FIS1 as an independent prognostic factor for OS in patients with oral melanomas in both univariate and multivariate models. Most importantly, such data suggest that the FIS1 plays an essential part in the pathogenesis of cutaneous and oral melanomas, and it is consequently appointed as a possible prognostic biomarker for these tumors, mainly for oral melanomas.

The exact role of MFN2 in cancer is not well established, as contradictory data, in some studies, have demonstrated an oncogenic activity, whereas other studies have researched its antioncogenic effect [13,32]. In this study's cohort of H&N melanomas, the MFN2 expression was associated with lower DFS and OS in the multivariate model for patients with CMs. Furthermore, high MFN2 expressions demonstrated a correlation with high risk for distant metastasis in a multivariate model. In oral melanomas, cases with high MFN2 expression have better prognosis than cases with lower expression. Although these data were not significant, it is important to highlight a possible MFN2 antioncogenic effect in these particular tumors. This corroborates with a previous study, in which B16F10 melanoma cell 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, which is validated by other studies [13,15]. Additional studies to determine the role of MFN2 in different human cancers types are highly suggested.

The current understanding of the clinical and pathological characteristics of MMs is mainly based on the analysis of individual cases or small series [33-35]. However, in this study, 56 oral and sinonasal melanoma samples were collected with strict follow-up and complete clinicopathological data. Within clinicopathological parameters for oral melanomas, worse outcome was identified for lesions in the palate and demonstrating a prognostic value of AMT and FIS1 for these tumors. In a univariate model, variables such 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 predictor for oral melanomas. Regarding the sinonasal melanomas, only an epithelioid/fusiform cellular morphology was identified as an independent prognostic factor. In contrast, in the univariate model, vascular invasion, and MFN2 were correlated with lower OS probability. The lack of predictive value of these clinicopathological 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 nasopharynx.

In conclusion, high mitochondrial content and FIS1 expression are associated with the aggressive characteristics and with

poor prognosis in patients with oral melanomas. Furthermore, high mitochondrial content predicted lower DFS in patients with CMs, as well as MFN2 that predicted distant metastasis in CMs of the H&N region. Altogether, the novel identified mitochondrial markers in this study may help in the development of new drugs that target the mitochondria and modulate the mitochondrial dysfunction that underlies in the pathogenesis of mucosal and CMs to prevent tumor recurrence and distant metastasis.

Author contributions

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Supplementary data

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