Immunohistochemical Expression of p16, p21, p27 and Cyclin D1 in Oral Nevi and Melanoma

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

Keywords Oral melanoma · Oral nevi · Immunohistochemistry · Cell cycle

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Introduction

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

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

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

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

^a Santa Cruz, Santa Cruz, California, USA^b Dako, Dako Corporation, Carpinteria, California, USA

immunohistochemistry the expression of p16, p21, p27 and cyclin D1 in oral melanocytic nevi and melanoma.

Materials and Methods

Formalin-fixed, paraffin-embedded tissue blocks were obtained from 51 oral melanocytic lesions, corresponding to 38 intramucosal nevi and 13 primary oral melanomas. All lesions were assessed using H&E preparations to

Table 2 Characteristics of intramucosal nevi and immunohistochemical findings

Case	Sex	Age (years)	Location	p16 (%)	p21	p27 (%)	Cyclin D1
01	F	25	Hard palate	70	0	65	0
02	F	35	Buccal mucosa	80	0	80	0
03	F	41	Not specified	75	0	75	0
04	M	16	Hard palate	70	0	80	0
05	F	32	Buccal mucosa	85	0	85	0
06	M	26	Buccal mucosa	70	0	75	0
07	F	27	Hard palate	70	0	80	0
08	F	45	Buccal mucosa	80	0	65	0
09	F	Not specified	Hard palate	75	0	85	0
10	F		Gingiva	80	0	90	0
11	M	23	Hard palate	80	0	85	0
12	M	29	Not specified	70	0	80	0
13	F	67	Buccal mucosa	80	0	65	0
14	F	49	Buccal mucosa	90	0	80	0
15	F	25	Hard palate	75	0	75	0
16	M	28	Not specified	80	0	70	0
17	F	26	Gingiva	75	0	90	0
18	M	34	Buccal mucosa	80	0	65	0
19	F	28	Hard palate	70	0	75	0
20	F	40	Gingiva	75	0	80	0
21	F	33	Buccal mucosa	70	0	70	0
22	F	35	Hard palate	80	0	80	0
23	F	28	Gingiva	70	0	75	0
24	M	37	Hard palate	90	0	65	0
25	F	39	Buccal mucosa	80	0	70	0
26	F	Not specified	Gingiva	70	0	70	0
27	F		Hard palate	85	0	90	0
28	F	37	Gingiva	85	0	65	0
29	F	32	Buccal mucosa	70	0	80	0
30	F	31	Hard palate	80	0	80	0
31	M	38	Gingiva	80	0	70	0
32	F	29	Gingiva	75	0	80	0
33	F	30	Hard palate	70	0	80	0
34	F	35	Gingiva	85	0	80	0
35	F	31	Buccal mucosa	70	0	75	0
36	F	29	Hard palate	80	0	75	0
37	F	34	Gingiva	75	0	70	0
38	F	30	Not specified	85	0	80	0

F female, M male

Table 3 Characteristics of primary oral melanomas and immunohistochemical findings

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

F female, M male, HP hard palate

Fig. 1 Expression of p16 in oral melanocytic lesions. As we used DAB to develop the reactions, the color in the photographs can be confounded with melanin in the cytoplasm of benign and malignant melanocytes, but this did not interfere with analyses of the results, as we used only nuclear markers. **a, b** Intramucosal nevi showing diffuse strong nuclear expression of p16 in melanocytic cells nests,

c malignant cells of invasive oral melanoma negative for p16. Note that cells of the stratum spinosum of normal oral epithelium was positive, serving as an internal positive control, **d** one case out of 13 cases of melanoma expressed nuclear p16 in the neoplastic cells, as shown here (immunoperoxidase, **a, c** $\times 200$; **b, d** $\times 400$)

Fig. 2 Expression of p21 in oral melanocytic lesions: **a** intramucosal nevi showing expression of nuclear p21 only in the epithelial cells of the normal oral mucosa, serving as internal control, **b** melanocytes of

intramucosal nevi showing absence of p21 nuclear expression, **c**, **d** invasive oral melanoma expressing moderate nuclear positivity for p21 in the tumor cells (immunoperoxidase, **a**, **c** $\times 200$; **b**, **d** $\times 400$)

confirm the diagnosis. Intramucosal nevi patients included 30 women and eight men, aged 16–67 years, while the primary oral melanomas corresponded to nine women and four men, aged 23–86 years. Diagnosis of melanoma was confirmed by clinical and histological characteristics, excluding the presence of melanoma at other anatomical sites and consequently the possibility of oral metastasis. Melanomas were histologically classified according to Prasad et al. [11]; 11 and two cases corresponded to level III (very deep invasion) and in situ, respectively.

For immunohistochemical staining, 3 μ m thick sections mounted on silane-coated glass slides were used. Briefly, the sections were deparaffinized and rehydrated in graded ethanol solutions. After antigen retrieval with EDTA/Tris buffer (pH 9.0) in a microwave oven (1380 W; Panasonic, São Paulo, Brazil), endogenous peroxidase activity was blocked with 20% H_2O_2 using five cycles of 5 min each. Overnight incubation with the primary antibodies for p16,

p21, p27 and cyclin D1 (Table 1) diluted in bovine serum albumin (BSA) was followed by incubation with the secondary antibody conjugated with polymer dextran marked with peroxidase (Dako EnVision Labeled Polymer; Dako, Glostrup, Denmark). Reactions were developed with a solution containing 0.6 mg/ml 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma, St. Louis, MO, USA) and 0.01% H_2O_2 and counterstained with Carazzi's hematoxylin. Positive and negative controls were included in all reactions. Only nuclear staining was considered positive. As we used DAB as the developer, the reactions could be confounded by melanin in the cytoplasm of benign and malignant melanocytes, but this was not a problem for interpretation as we used only nuclear markers. Immunohistochemical results were quantified considering the percentage of cells expressing nuclear positivity, counting 1,000 cells per slide. Immunoreactive cells were counted randomly with a minimum of 10 high-power fields ($\times 400$)

Fig. 3 Expression of p27 in oral melanocytic lesions: **a, b** intramucosal nevi showing strong p27 expression in the nuclei of melanocytic cell nests, **c, d** invasive oral melanoma presenting weak to moderate

nuclear expression of p27 in few malignant cells (immunoperoxidase, **a, c** $\times 200$; **b, d** $\times 400$)

according to Stefanaki et al. [6]. The staining intensity was subjectively classified as weak (+), moderate (++) or strong (+++).

Results

The immunohistochemical results of the 51 oral melanocytic lesions are listed in Tables 2 and 3. p16 was strongly expressed in more than 70% of cells in all cases of intramucosal nevi, while only one oral melanoma case was positive in 35% of neoplastic cells, mainly in the central part of the tumor (Fig. 1). p21 was negative in all intramucosal nevi, but epithelial cells of the normal oral mucosa were positive in the stratum spinosum, serving as an internal positive control. On the other hand, p21 was expressed in all oral melanomas investigated, showing weak to moderate nuclear expression in 10–50% of tumor cells, mainly on the superficial central compartment of the

tumor rather than the tumor margins (Fig. 2). p27 was strongly expressed in all nevi, with more than 65% of cells positive. Six out of 13 cases of oral melanomas were positive for p27, but with only 5–20% of the cells being positive, weakly or moderately. The labeling was heterogeneous, not involving preferential areas (Fig. 3). Cyclin D1 was negative in all nevi, and only normal epithelial cells of the stratum spinosum were positive. All oral melanomas showed cyclin D1 positivity in 10–40% of the tumor cells, mainly in the invasive tumor component, with moderate to strong staining intensity (Fig. 4).

Discussion

Melanoma is known to exhibit aberrant expression of cell cycle-regulating genes, and these abnormalities at the G1/S checkpoint are considered crucial steps in the genesis and progression of melanoma [12, 13]. In this study, we

Fig. 4 Expression of cyclin D1 in oral melanocytic lesions: **a** intra-mucosal nevi showing nuclear cyclin D1 expression in epithelial cells of the normal oral mucosa, serving as an internal positive control, while the nevi were negative, **b** intra-mucosal nevi showing absence of

cyclin D1 nuclear expression, **c, d** invasive oral melanoma showing cyclin D1 positivity in neoplastic cells (immunoperoxidase, **a, c** $\times 200$; **b, d** $\times 400$)

evaluated the immunohistochemical expression of the cell cycle regulators p16, p21, p27, and cyclin D1 in 38 intra-mucosal nevi and 13 primary oral melanomas. There are no data on the immunohistochemical expression of these proteins in intra-mucosal nevi and only two reports considering p16 expression in oral melanomas [1, 14].

The negative cell cycle regulators p16 and p27 were diffusely expressed at high levels in all intra-mucosal nevi cells, in contrast to oral melanomas, which demonstrated a much lower expression of these proteins. In agreement with these results, cutaneous nevi have been shown to be uniformly positive for p16 in more than 70% of cells [7, 15]. On the other hand, loss of p16 expression has also been demonstrated in almost 50% of primary cutaneous and oral melanomas, similarly to many other cancers, including pancreatic, esophageal, lung, head and neck, breast, bladder, brain and ovarian [1, 5, 13, 14]. Although there is general consensus regarding the loss of p16 expression in

cutaneous melanomas, it is suggested that this occurs in later stages of the disease, as it is not altered in melanoma in situ [16]. However, these findings may not be applied to oral melanomas, because we found reduced p16 expression also in oral in situ melanomas, suggesting that this alteration may be relevant in the initial phases of oral melanoma development. In oral nevi, expression of p16 was not altered, indicating that in the mouth, nevi do not precede melanoma formation [5]. Reduced expression of p16 in cutaneous melanomas is also associated with a significant risk of recurrence and it is considered an independent predictor for decreased patient survival [13, 17, 18].

p27 was strongly expressed in all nevi cells, in contrast to oral melanomas where six out of 13 cases were weakly or moderately positive. High expression of p27 is a feature of benign lesions, and it can be used to distinguish benign and malignant tumors [5]. Strong p27 nuclear staining has been shown in cutaneous nevi, including Spitz nevi [9, 19],

and its expression is progressively lost during the development of melanomas [9, 13, 19]. There are few studies reporting the association of p27 with clinical outcome in melanoma patients. Flørenes et al. [20] found that decreased expression of p27 was significantly associated with increasing Breslow thickness and reduced disease-free survival in primary nodular melanomas. However, other authors have reported that p27 nuclear staining was not associated with patient prognosis [19].

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

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

It is important to note that oral melanoma is a rare lesion usually diagnosed at advanced stages and, compared to its cutaneous counterpart, some differences are observed. In fact, oral melanomas differ from cutaneous ones in several aspects, including risk factors, such as actinic radiation, family history, and an association with

atypical nevi—factors applied mainly to cutaneous melanoma [27]. Genetic events are also distinct from those of its cutaneous counterpart. Mutation and up-regulation of c-KIT has been identified in both oral and cutaneous melanomas, and may activate downstream molecules such as RAS and RAF, thus leading to tremendous cell proliferation [28]. BRAF mutations are classical events in cutaneous melanomas, present in two-thirds of these melanomas, while in oral melanoma these mutations are very rare [28, 29]. Those factors, in association with our results, make oral melanoma unique from cutaneous melanomas.

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

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