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Evaluation of the expression of Bmi-1 stem cell marker in sinonasal melanomas and its correlation with the expression of cell cycle proteins

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Abstract

Background: Sinonasal melanomas (SNM) are aggressive neoplasms, which present distinct clinicopathological and molecular aspects when compared to cutaneous melanomas (CM). B-cell-specific moloney murine leukemia virus integration site-1 (Bmi-1) is a stem cell marker involved in the regulation of the cell cycle and has been found to be expressed in 70% of CM and 100% of benign nevi. Regarding the cell cycle, Bmi-1 is known to be an upstream repressor of p16, which is a tumor suppressor encoded by the INK4a/Arf locus. Considering this, the aim of this study is to evaluate the immunohistochemical expression of Bmi-1 in a series of SNM and its correlation with the expression of cell cycle proteins (p16 and Ki-67, a nuclear antigen of proliferating cells).

Methods: In 16 cases of SNM, nuclear expression of Bmi-1 and nuclear and cytoplasmic of p16 was classified as: absent, low (> 5 to < 50% of cells) and high (≥50%). Ki-67 proliferation index was represented by the ratio positive cells/ total cells.

Results: Histologically, all cases presented varying amount of necrosis and 75% contained undifferentiated cells. Bmi-1 was detected in 6 cases (37.5%) with high level of expression in 2; p16 expression was seen in 10 cases (62.5%) with high level in 7. The frequency of p16 expression did not differ significantly between tumors with or without Bmi-1 expression. Ki-67 index ranged from 8 to 22%. Neither Bmi-1 nor p16 expression showed correlation with Ki-67 index. Bmi-1 negative tumors presented more extensive necrosis (71.4%); no association between Bmi-1 expression and undifferentiated phenotype was observed.

Conclusions: In our SNM series, low immunohistochemical expression of Bmi-1 was a common phenomenon favoring the hypothesis that mucosal melanoma possibly presents molecular pathways different from the cutaneous counterpart. In SNM, Bmi-1 and p16 expression levels did not correlate with each other or with the cell proliferative index.

Keywords: Sinonasal melanoma, Bmi-1, p16, Immunohistochemical expression

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Background

The sinonasal region is the major site of mucosal melanoma of the head and neck (Williams, 2017). Sinonasal melanomas (SNM) are rare, their etiology is poorly understood, and they present distinct clinicopathological and molecular aspects when compared to their cutaneous counterpart, which is etiologically related to ultraviolet radiation (Franchi et al., 2006). SNM are associated with poor overall survival at 5 years, frequent undifferentiated phenotype and higher rates of mutations involving C-Kit (CD 117) pathway (López et al., 2016). These particularities show the need to deepen knowledge about SNM, in order to better understand its pathogenesis, which is important for the development of therapeutic strategies.

It is known that in malignant tumors a subgroup of neoplastic cells expresses proteins that are considered stem cell (SC) markers. Neoplastic SCs are capable of self-renewal and differentiation; in addition, they have been considered to play an important role in tumor initiation and progression as well as in therapeutic resistance (Siclari & Qin, 2010; Rangwala et al., 2011; Schatton et al., 2008). Among the SC markers, B-cell-specific moloney murine leukemia virus integration site-1 (Bmi-1) is a transcription factor involved in the regulation of the cell cycle and apoptosis. Deregulation of Bmi-1 expression has been described in several types of cancer and associated with histological features of tumor aggressiveness (increased number of mitotic figures and necrosis) as well as with tumor biological behavior (metastatic capacity, predictor of prognosis) (Bachmann et al., 2006; Vrzalikova et al., 2008; Song et al., 2006; Liu et al., 2008; Mihic-Probst et al., 2007; Silva et al., 2007; Bonora et al., 2015) P16 is a tumor suppressor protein, cyclin dependent kinase inhibitor involved in cell proliferation pathways.(Li et al., 2006). As Bmi-1 is a suppressor of the Ink4a / Arf locus, which encodes p16, it has been proposed that this interaction could lead to increased cell proliferation, affecting the biological behavior of the tumor (Song et al., 2006; Mihic-Probst et al., 2007; Allegra et al., 2012; Vormittag et al., 2009; Chen et al., 2011).

Drawing from this background, the aim of our study was to evaluate the immunohistochemical expression of Bmi-1 in a series of SNM and its correlation with the expression of cell cycle proteins (p16 and Ki-67, a nuclear antigen of proliferating cells). To the best of our knowledge, expression of Bmi-1 in SNM has yet to be determined.

Material and methods

This study was approved by the Institutional Ethics Committee. The surgical pathology archives of the Hospital of the University of Campinas (UNICAMP), São Paulo-Brazil, were reviewed between 1990 and 2016 and contained 16 tumors which had been diagnosed as SNM and had available slides and/ or blocks. All cases were reviewed to confirm the diagnosis and clinical details were obtained from medical records.

Immunohistochemistry

Immunohistochemical studies were performed on sections from representative formalin fixed paraffin embedded blocks. All cases were stained with antibodies showed in the Table 1. Immunoreactivity for Bmi-1 and p16 was assessed and classified as absent (0 to 5%), low (> 5 to < 50% of cells) and high (≥50%) according to Mihic-Probst et al.(Mihic-Probst et al., 2007). For Bmi-1, only nuclear staining was considered positive whereas for p16, both nuclear and cytoplasmic reactivity was used; high p16 expression could be nuclear and cytoplasmic or exclusively cytoplasmic in ≥50% of tumor cells. Ki-67 nuclear immunohistochemical assessment was performed with the help of Aperio ImageScope nuclear algorithm (Aperio ScanScope; Aperio Technologies, Vista, Calif). Five high-power fields were randomly selected (original magnification × 200), and 3000 tumor cells per slide were counted approximately. The labeling index for Ki-67 in each case of nasal melanoma was expressed as a percentage of positive tumor cells.

Statistical analysis

A Student's T test was used for comparison of the quantitative variables. Mann-Whitney U test was used for comparison of the numeric variables between the groups as appropriate. Data were presented as mean ± SD (standard deviation), and the results with $p < 0.05$ were considered significant. All the statistical procedures were performed using Graph Prism version 6.0 for Mac (GraphPad Software® La Jolla, USA).

Results

Clinicopathologic findings

Table 2 shows the clinicopathological findings of 16 cases of SNM. In all cases but one, the nasal cavity was the site of origin of the tumor and all were advanced lesions, i.e., they invaded submucosa and deep soft tissue (T3/ T4 in the TNM classification). The median age of the patients was 62.2 years (range, 24–78 years) and 60% were women. Histologically, growth pattern of the solid type was observed in almost all cases and most of them presented areas with peritheliomatous arrangement of

Table 1 Antibodies used in this study

ANTIGEN	CLONE	DILUTION	SOURCE
P16	Polyclonal	1:100	Dako
Bmi-1	DC9	1:300	Millipore
Ki-67	MIB-1	1:100	Dako

Table 2 Clinicopathological findings of 16 cases of sinonasal melanoma

Cases	Clinical findings			Predominant cellular composition	Melanogenesis	Necrosis	Vascular invasion	Neural invasion
	Gender	Age	Origin					
1	–	–	NA-	undifferentiated	0	< 50%	Negative	Negative
2	male	69	Nasal cavity	Epithelioid	< 50%	< 50%	Negative	Negative
3	female	71	Nasal cavity	undifferentiated	< 50%	< 50%	Positive	Negative
4	female	24	Maxillary sinus	Spindle cell	> 50%	< 50%	Negative	Negative
5	male	65	Nasal cavity	Epithelioid	0	> 50%	Negative	Negative
6	female	67	Nasal cavity	undifferentiated	< 50%	< 50%	Negative	Negative
7	female	60	Nasal cavity	undifferentiated	0	> 50%	Negative	Negative
8	male	66	Nasal cavity	undifferentiated	0	> 50%	Negative	Negative
9	male	55	Nasal cavity	Spindle cell	0	< 50%	Positive	Negative
10	female	58	Nasal cavity	undifferentiated	0	> 50%	Negative	Negative
11	female	67	Nasal cavity	undifferentiated	< 50%	< 50%	Negative	Negative
12	male	66	Nasal cavity	undifferentiated	0	> 50%	Negative	Negative
13	female	50	Nasal cavity	Epithelioid	> 50%	> 50%	Negative	Negative
14	female	78	Nasal cavity	undifferentiated	0	> 50%	Negative	Negative
15	male	65	Nasal cavity	undifferentiated	0	> 50%	Negative	Negative
16	female	78	Nasal cavity	undifferentiated	< 50%	> 50%	Negative	Negative

tumor cells (Fig. 1). Tumor cellular composition varied but 75% of cases contained undifferentiated cells (Fig. 1). Neoplastic cells with melanin were seen in 43% of cases. All tumors exhibited areas of necrosis, which was extensive (> 50% of tumor area) in 56.2% (9/16) of cases. Vascular invasion was observed in two cases, but neural infiltration was not seen. However, it should be noted that most of cases were incisional biopsies.

Immunohistochemical findings

In SNM, the nuclear expression of Bmi-1 was detected in 6 cases (6/16–37.5%), with high level of expression in 2 of them (Fig. 1). Nuclear and cytoplasmic p16 expression was seen in 11 cases (11/16–68.7%), which presented high level of expression in 7 (7/16–43.7%) and low in 4 (4/16–25%). P16 expression was absent in 5 cases (5/16–31.2%) (Fig. 1). The frequency of p16 expression did not differ significantly between tumors with or without Bmi-1 expression (66.6 and 60% of cases were p16 positive, respectively) ($p = 0.84$). The combination high Bmi-1 and low expression ratio of p16 was noted in only 1 of 16 cases. Regarding the morphological aspects associated with tumor aggressiveness (Table 3), most tumors with extensive necrosis were Bmi-1 negative (71.4%). However, Bmi-1 expression was not related to the undifferentiated cellular pattern; Bmi-1 positive and negative tumors showed a similar frequency of lesions composed predominantly of undifferentiated cells. The Ki-67 proliferation index ranged from 8 to 22% and no significant difference was detected between tumors with and without Bmi-1 expression (means 17.3% versus 15%) ($p = 0.33$) and the ones with or without p16 expression (means 16.3% versus 14.7%, respectively) ($p = 0.83$).

Discussion

Overexpression of Bmi-1 has been described in several malignant tumors and related to tumorigenesis, metastasis, and increased resistance to ionizing radiation (Song et al., 2006; Allegra et al., 2012; Vormittag et al., 2009; Chen et al., 2011). Furthermore, Bmi-1 has been found as a predictor of prognosis in breast, gastric, nasopharyngeal and salivary adenoid cystic carcinomas (Bachmann et al., 2006; Vrzalikova et al., 2008; Song et al., 2006; Liu et al., 2008; Mihic-Probst et al., 2007; Silva et al., 2007). However, in melanoma, studies on Bmi-1 expression have shown conflicting results. Bachmann et al .(Bachmann et al., 2008) reported that in invasive melanomas, the loss of Bmi-1 expression was associated with increased cell proliferation, necrosis, and decreased patient survival. In contrast, Mihic-Probst et al.(Mihic-Probst et al., 2007) suggested that the increase of Bmi-1 expression could induce a metastatic tendency in cutaneous melanoma. Interestingly, recently, experimental studies have reinforced Mihic–Probst et al.(Mihic-Probst et al., 2007) findings in human melanoma. In these, downregulation of Bmi-1 was shown to inhibit the aggressive behavior of melanoma cells by reversing epithelial-mesenchymal transition,(Liu et al., 2017) whereas Bmi-1 levels increased with tumor progression, promoting all the steps of the metastatic cascade (Ferretti et al., 2016).

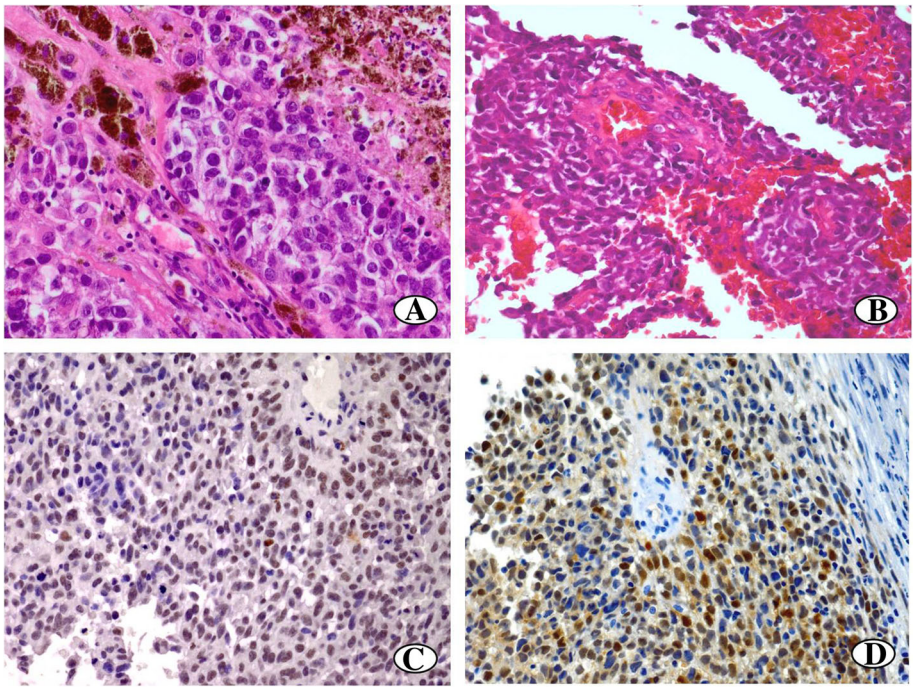

Fig. 1 Sinonasal melanoma: **a** and **b** – histopathological features: tumor composed of small undifferentiated cells (**a**), which form a cuff surrounding a blood vessel (peritheliomatous arrangement). **c** and **d** – immunohistochemical findings: nuclear expression for Bmi-1 (**c**) and nuclear and cytoplasmic expression for p16 (**d**) are seen in most tumor cells

To the best of our knowledge, this is the first time that Bmi-1 is analyzed in SNM; we detected expression of Bmi-1 in 37.5% of the tumors and in only 12.5% of cases (2/16) the protein was highly expressed (at least 50% of cells). These findings indicate that SNM presents a markedly low Bmi-1 expression when compared to cutaneous melanocytic lesions; 60–70% of cutaneous melanoma and 100% of benign nevi present high levels of Bmi-1 expression (at least 50% of cells) (Mihic-Probst et al., 2007; Bachmann et al., 2008). Therefore, our findings reinforce the idea that SNM exhibits a different immunophenotype from cutaneous melanoma, since in SNM absence or low Bmi-1 expression appears to be a usual phenomenon. On the other hand, as Bmi-1 expression is believed to promote stem cell state in tumor cells,(Cao et al., 2011) our results suggest that SNM usually contains a small subpopulation of Bmi-1 positive cancer stem cell. Of interest, overexpression of Bmi-1 has been reported to correlate with therapy failure (Cao et al., 2011). Thus, it is likely that cutaneous and SNM may require different therapeutic strategies given their significant differences in quantity of Bmi-1 positive cancer stem cell. Deserves comments that the role of stem cells in neoplasia is complex and has been subject of numerous studies. In malignant melanoma, besides Bmi-1 other markers of cancer stem cells have been described, such as CD166, CD133, nestin and CD44 (Regauer et al., 1999; Klein et al., 2007; Zhu et al., 2018). Interestingly, CD44 (a transmembranous adhesion molecule) has also been investigated in SNM.(Regauer et al., 1999; Zhu et al., 2018) Both Bmi-1 and CD44 have been reported to be highly expressed in benign melanocytic lesions, (Mihic-Probst et al., 2007; Bachmann et al., 2008; Harwood et al., 1996) but differently from Bmi-1, CD44 has been detected in a large proportion of cells of invasive SNM (Regauer et al., 1999; Zhu et al., 2018). These diverse features of the markers with stem-like properties reinforce that their roles within a melanoma-associated network still need to be better clarified.

Table 3 Frequency of undifferentiated phenotype, necrosis and Ki-67 proliferation index in tumors with and without Bmi-1 expression

	Undifferentiated phenotype	Necrosis		Proliferation index
	(12 cases)	> 50% (7 cases)	< 50% (9 cases)	Means
Bmi 1+	6/12 (50%)	2/7 (28,5%)	4/9 (44,4%)	17,3%
Bmi 1-	6/12 (50%)	5/7 (71,4%)	5/9 (55,5%)	15%

In SNM, invasion into deep tissue, undifferentiated cells comprising > 25% of tumor, necrosis, and vascular invasion have been considered features of tumor aggressiveness and predictors of poor prognosis (Gnepp, 2009). In the current series, we did not find any association of Bmi-1 expression with these aggressiveness features, except for tumors without Bmi-1 expression, which presented higher frequency of extensive tumor necrosis (> 50 of the lesion). This relationship has previously been noted in cutaneous melanoma as well (Bachmann et al., 2008). In addition, our results confirm those reported by other authors (Williams, 2017) showing that SNM are neoplasms more undifferentiated than their cutaneous counterpart. They were frequently composed of undifferentiated small cells and most of them did not show melanogenesis, which was seen in 43% of cases. From the diagnostic point of view, it is important to recognize SNM as a neoplasm often constituted by small round cells, as this region is affected by other tumors that share this morphology, such as lymphoma, Ewing's sarcoma, olfactory neuroblastoma, and rhabdomyosarcoma (World Health Organization, 2017). Therefore, in the diagnostic evaluation of an undifferentiated neoplasia composed of small round cells of the sinonasal region, it is necessary to include markers that identify melanocytic lesions (S-100 protein, HMB45 and Melan A) in the immunohistochemical panel.

In non-neoplastic tissues, Bmi-1 is a transcription factor and epigenetic regulator essential for maintaining the repression of genes involved in cell proliferation. The effect of Bmi-1 on cell proliferation is partially mediated through repression of the locus encoding p16,(Huber et al., 2011) which is one of the proteins responsible for controlling the G1-S transition of the cell cycle (Li et al., 2006). The p16 protein inhibits the formation of the cyclin D1/cdk4/6 complexes required for the phosphorylation of Rb and consequently the cell cycle progression is slowed down or blocked (Li et al., 2006; Fecher et al., 2009; Mitra & Fisher, 2009.) Therefore, repression of p16 by Bmi-1 leads to progression of the cell cycle. However, in malignant tumors, the expression of Bmi-1 does not seem to be necessarily linked to p16 expression or to have reflection on cell proliferation. For example, Bmi-1 expression has been described to have no impact on cell proliferation in lung, colon / rectum and brain cancers or correlation with p16 expression in head and neck carcinomas (Vonlanthen et al., 2001; Breuer et al., 2004; Kim et al., 2004; Hemmati et al., 2003; Lundberg et al., 2016). In melanoma, there are few studies on the relationship between Bmi-1 and cell proliferation and they showed conflicting results. In cutaneous melanoma, loss of Bmi-1 expression was found to be associated with increased tumor cell proliferation whereas experimental studies have shown that Bmi-1 had no effect on proliferation or tumor growth (Bachmann et al., 2008; Ferretti et al., 2016).

In SNM, this is the first time that the possible relationship between the levels of expression of Bmi-1 and p16 as well as their connections with cellular proliferation has been analyzed. In our series no association between Bmi-1 expression and p16 status was detected; the expected combination high Bmi-1 and low expression of p16 was rarely observed (only 1 case). Furthermore, Ki-67 proliferation index was similar in tumors with or without expression of Bmi-1 or of p16. Thus, our findings in SNM reinforce those detected by Ferretti et al. (Ferretti et al., 2016) in melanoma cells, where Bmi-1 levels had no influence on its proliferative capacity. In addition, our results also suggest that Bmi-1 does not suppress p16 expression in SNM. This phenomenon has been observed in cutaneous melanoma as well, leading to the hypothesis that in melanoma, Bmi-1 might exert its action in a p16 independent manner (Bachmann et al., 2008). Indeed, the loss of p16 expression has been described to occur in mucosal melanomas in up to 50% of cases(López et al., 2016) and in our series of SNM such event was found in 31.2% of cases.

Conclusion

In our SNM series, low immunohistochemical expression of Bmi-1 was a common phenomenon favoring the hypothesis that mucosal melanoma possibly presents molecular pathways different from the cutaneous counterpart. In SNM, Bmi-1 and p16 expression levels did not correlate with each other or with the cell proliferative index, suggesting that their immunohistochemical expressions might reflect other functions diverse from those seen in non-neoplastic tissue.

Abbreviations

Bmi-1: B cell specific moloney murine leukemia virus site 1 integration; CM: Cutaneous Melanomas; SC: Stem Cells; SNM: Sinonasal Melanomas

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Availability of data and materials

The datasets used and analyzed during this study are available from the corresponding author on reasonable request.

Authors' contributions

Conception and design of study: FVM, AA. Selection of cases: HTS, JSN, FM. Histological classification: HTS, JSN, FVM, AA. Acquisition of clinicopathological data: HTS, JSN, EAE, FM. Immunohistochemical reactions: HTS, JSN, JFS, EAE. Cells counting: FPS. Analysis and/or interpretation of results: HTS, FVM, AA. Statistical Analysis: VAM. Drafting the manuscript: HTS, FVM, AA. Revising the manuscript critically for important intellectual content: FVM, AA. Approval of the version of the manuscript to be published: HTS, JFS, FM, EAE, JSN, VAM, FPF, FVM, AA. All authors read and approved the final manuscript.

Ethics approval and consent to participate
This study was approved by the Institutional Ethics Committee from Faculty of Medical Sciences – UNICAMP.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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