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Evaluation of Kindlin-1 and Ki-67 immunohistochemical expression in primary cutaneous malignant melanoma: a clinical series

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Abstract

Background: The capacity for prognostic prediction of cutaneous melanoma, one of the most aggressive cancers, is still difficult due to the tumor heterogeneity and lack of reliable tumor markers. The objective of this study is to correlate, through immunohistochemistry, a Ki-67 and Kindlin-1 staining in malignant melanomas with the prognosis of the disease.

Methods: A historical cohort study. Immunohistochemistry, using mouse anti-human Kindlin-1 and Ki-67 monoclonal antibodies, was performed using tissue blocks from primary cutaneous melanoma patients treated between 2006 and 2014 at our institution. Information regarding pathological data and outcomes were retrieved from medical records. Statistical analyses were conducted in SPSS version 18.0.

Results: Thirty patients were included. The median age was from 50.93 ± 15.31 years old. The expression of Ki-67 was detected in all patients with primary cutaneous melanoma, while Kindlin-1 was negative in two. Kindlin expression was not significantly correlated with Ki-67 expression by Spearman's rank correlation analysis ($P = 0.46$), as well as the expression of both markers and the clinical stage ($P = 0.34$ and 0.18, respectively). Breslow, Clark and mitotic rate were significantly correlated with AJCC stage ($P = 0.001$).

Conclusion: Other studies investigating clinical evolution are needed to further test the potential of these markers as possible prognostic markers.

Keywords: Melanoma, Kindlin, Ki-67; biomarkers, Immunohistochemistry

Introduction

Cutaneous malignant melanoma is a tumor originating from skin melanocytes, and its incidence in individuals of European origin continues to rise worldwide. In 2016, approximately 76,380 new cases were diagnosed in the United States [1]. In addition, there are approximately 3.03 and 2.59 new cases per 100,000 people for men and women, respectively, in Brazil. The three southernmost states of Brazil have the highest proportion of individuals of Northern European Caucasian ancestry, with approximately 6.96 new cases per 100,000 men and 6.5 per 100,000 women in 2016 [2, 3]. Melanomas are one of the most aggressive malignant neoplasms. Moreover, the clinical course is often unpredictable, and despite improved options due to targeted therapies, metastatic melanoma is difficult to treat [4–6].

The staging system proposed by the American Joint Committee on Cancer (AJCC) is currently used for melanoma classification, prognostic prediction and patient management [7]. Tumor invasion (Breslow thickness) has become the gold standard for stratifying patients according to the risk of metastatic disease. However, it has been noted that in some cases, tumor thickness is not an accurate indicator of biologic behavior [8]. For example,

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early-stage melanomas are clinically heterogeneous, with a subset exhibiting high-risk behaviors; approximately 5% of stage I melanomas metastasize early and eventually cause death [9]. Surgical excision of localized tumors (AJCC stages I and II) may definitively treat many patients. For these patients, the overall 5-year survival rate is approximately 80%, suggesting that nearly 20% of patients may have micrometastatic disease at the time of diagnosis [10].

Thus, identifying biomarkers for use in conjunction with traditional cancer staging and prognosis might improve early diagnosis and patient care. Despite efforts to date, reliable biomarkers are still lacking [11]. Nonetheless, a prognostic association has been shown between the mitotic index and thin melanoma [12], and the Ki-67 index has been studied as a prognostic marker. However, the prognostic value of proliferative activity in cutaneous malignant melanoma remains unclear. Indeed, there are divergent findings among studies about the use of the Ki-67 index or conventional prognostic factors (sex, location, level of invasion, tumor thickness, ulceration, mitotic count, prognostic index and clinical stage), and some studies indicate that the Ki-67 index does not offer additional prognostic information for localized cutaneous malignant melanoma [13].

Immunohistochemical biomarkers able to provide additional information about disease progression have been extensively evaluated [14]. Unfortunately, none have yet been incorporated into clinical practice. Some of these biomarkers have demonstrated statistical significance as prognostic markers in the research setting, though there has been no systematic analysis of molecular biomarkers in an attempt to identify those capable of refining subgroups for individual AJCC stages [15, 16].

Kindlin-1

Kindlins have been investigated as cancer biomarkers for more than a decade. *Fermitin-family-member*-1 (FERMT1, Kindlin-1) is an epithelial-specific regulator of integrin functions that is associated with Kindler syndrome, a genetic disorder characterized by skin blistering, atrophy, and photosensitivity. The possible role of Kindlin-1 in cancer remains unknown [17].

The most studied function of kindlins is their role in integrin activation. The term *integrin* was coined more than three decades ago to designate a family of cell-surface adhesion receptors, and the interrelationship between integrins and cancer pathology inevitably led to consideration of the role of kindlins in cancer. Within the last 5 years, more than 70 publications have linked kindlins, integrins and cancer, including descriptions of an association between kindlin-1, skin cancer, and downregulation of Kindlin-3 expression in melanoma cells, which promotes metastasis [18, 19]. Furthermore, Kindlin-1-deficient cells display a defect in B1 integrin activation and reduced cell proliferation, adhesion, polarity and motility. Most kindlin-related studies have focused on the relevance of kindlin to skin disease. Regardless, kindlins are also reported to have associations with a poor prognosis in both breast and lung adenocarcinoma, and studies have documented aberrant Kindlin-1 expression levels in cancers of epithelial origin. Patients who lack Kindlin-1 (Kindler Syndrome patients) may have an increased risk of squamous cell carcinoma [20].

Kindlin expression levels are reportedly elevated in 60% of lung cancers and 70% of breast cancers. Kindlins have also been associated with the pathology of glioma and pancreatic cancer [21, 22]. Although research has demonstrated a role for the Kindlin-1 antibody in the prognosis of other cancers, there are, to the best of our knowledge, no studies of this marker in primary melanoma.

Ki-67

The Ki-67 antigen was discovered in a study of specific nuclear antigens for monoclonal antibodies in Hodgkin cancer cells; the Ki-67 antigen is an important tool for quickly and reliably determining the proliferation rate of malignant tumor cells [5]. Ki-67 has been assessed with regard to diagnostic differentiation between a nevus and melanoma, and the Ki-67 proliferation index is higher for malignant melanocytic tumors than nevi and is higher in metastases than in primary melanomas. Despite these results, a low Ki-67 index for a malignant lesion does not eliminate the possibility of melanoma, as some malignant tumors proliferate at low rates, further demonstrating that more studies are needed [23, 24].

A previous study sought to investigate whether the Ki-67 index is able to prevent false-negative diagnoses in melanomas. The authors proposed that a high cellular proliferation rate may be suggestive of melanoma and that the Ki-67 proliferation index might help prevent misdiagnoses of melanomas initially classified as benign or dysplastic nevi [25]. By comparing the prognostic impact of phosphohistone H3/MART1, Ki67/MART1, and hematoxylin and eosin staining, another study found alternatives to conventional detection of mitosis based on hematoxylin and eosin staining in the first two stages of primary cutaneous melanoma [26].

The aim of this study was to correlate Ki-67 and Kindlin-1 immunohistochemical staining in primary malignant melanomas with prognostic factors.

Materials and methods

Patients and tissue samples

We retrieved data for thirty patients from the files of patients diagnosed and treated at our institution (Irmandade Santa Casa de Misericórdia de Porto Alegre

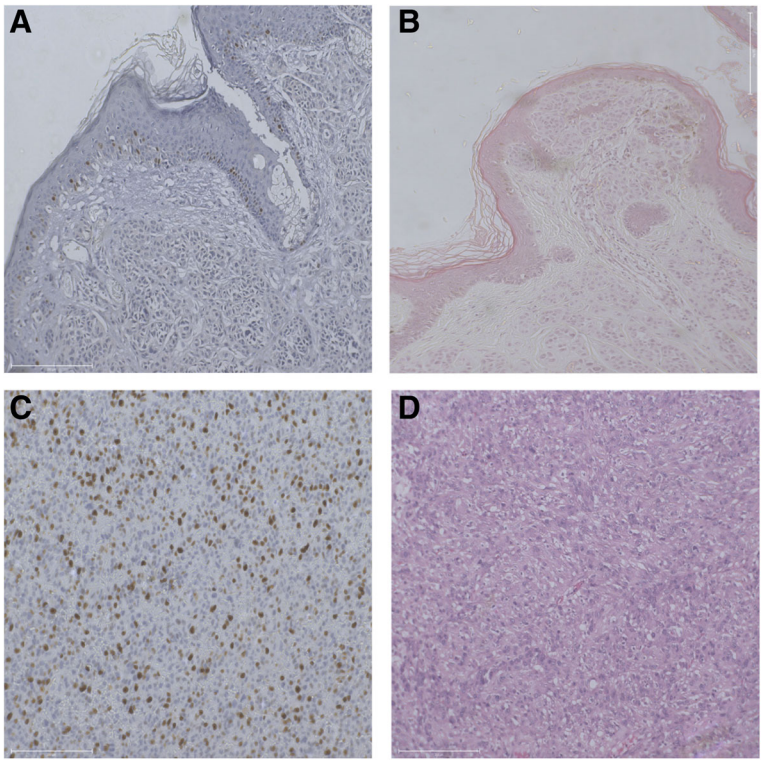

Fig. 1 H&E staining and Ki-67 expression by immunohistochemistry in primary cutaneous malignant melanoma (PCMM). The marker (brown) is expressed in the nuclei of PCMM cells. **a**: less than 1% of positivity and (**b**): corresponding to H&E staining; **c** greater than 60% of positivity and (**d**) corresponding to H&E staining

Hospital – ISCMPA, and Universidade Federal de Ciências da Saúde de Porto Alegre - UFCSPA) between July 2006 and September 2014; these patients all completed at least 2 years of follow-up. Clinical records were reviewed, and follow-up data were obtained from these and tumor registry records. A questionnaire about the main risk factors for the development of this neoplasia was created, and the following were recorded for the patients: sunburn episodes, familial and personal melanoma history, phenotypic characteristics and skin phototype.

Written informed consent was obtained from each patient according to the recommendations of the local ethics committee. This study was approved by the ethics committee of the hospital and the university where the study was developed (ISCMPA and UFCSPA, respectively).

For each patient, formalin-fixed paraffin-embedded tumor tissue (which was identified based on hematoxylin and eosin-stained sections and, when necessary, on S100 protein, melan A and HMB-45 immunohistochemistry sections) was obtained up from the pathology laboratory of ISCMPA. An independent histopathological review was performed by two pathologists on separate occasions.

Immunohistochemistry (IHC)

The most representative tumor area on the hematoxylin/eosin-stained slides was selected. Tissue sections 4-μm thick were placed on salinized slides and dried at 60 °C for 30 min. The slides were then deparaffinized by three washes in xylene (5 min each) and rehydrated by successive washes in ethanol (absolute, 95, 70 and 50%) and distilled water, according to our laboratory protocol. Antigen retrieval was performed in pH 9.0 TRIS-EDTA buffer for 40 min at 98 °C. After heating and cooling for 20 min, endogenous peroxidase activity was blocked by immersing the slides in 5% hydrogen peroxide in methanol (3 × 10 min). Finally, the slides were washed twice with 1X phosphate-buffered saline (PBS, pH 7.4) and incubated in 1% BSA (bovine serum albumin) solution to block non-specific protein binding.

Primary antibodies against Ki-67 (1:50, mouse anti-human monoclonal Mib-1 - Dako^{R}, California, USA) and Kindlin-1 (1:100, mouse anti-human monoclonal antibody - Millipore^{R}, Massachusetts, USA) were added and incubated for 1 h (room temperature), after which the slides were placed at 4 °C overnight. Colon and breast tissue were used as positive controls. The negative controls were the same tissues without primary antibody incubation. After washing with PBS, the slides were

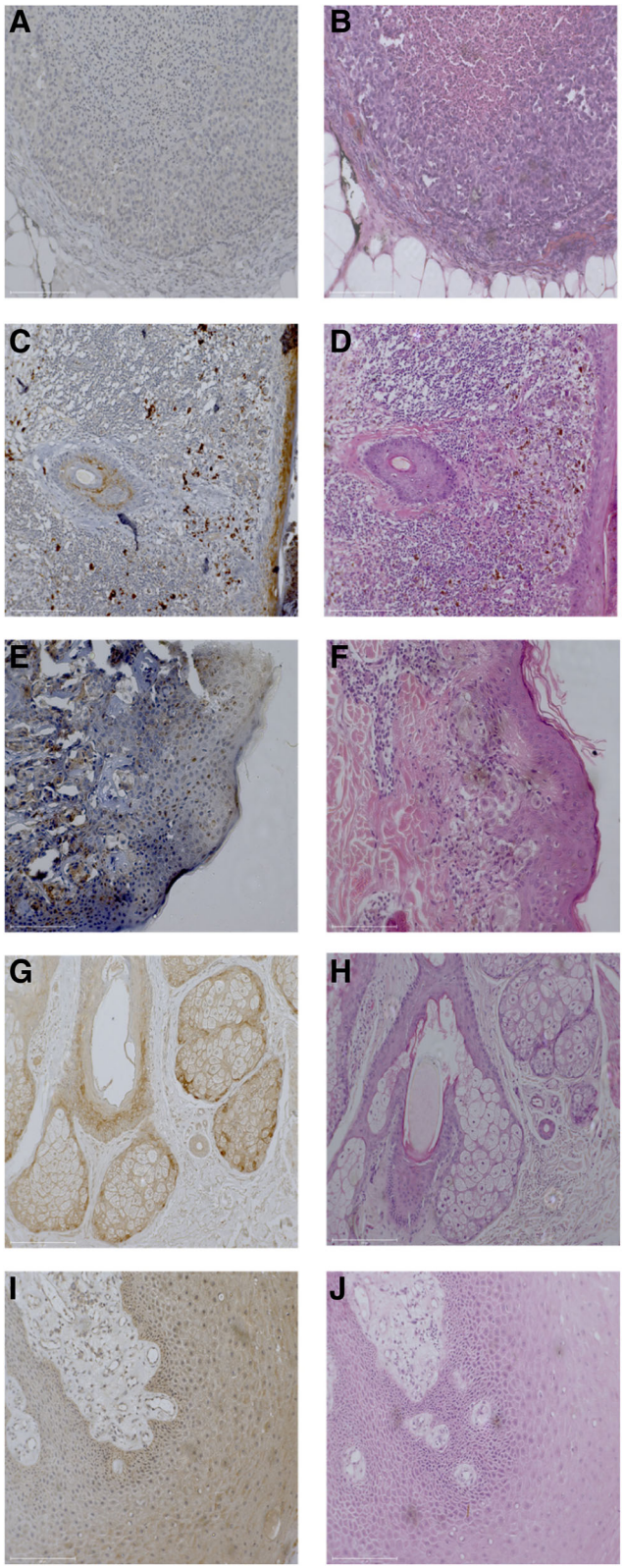

Fig. 2 (See legend on next page.)

(See figure on previous page.)
Fig. 2 H&E staining and kindlin-1 expression by immunohistochemistry in primary cutaneous malignant melanoma (PCMM). The marker (brown) is expressed in the citoplasm of PCMM cells. **a** negative and (**b**) corresponding to H&E staining; **c** focal and (**d**) corresponding to H&E staining; **e** multifocal and (**f**) corresponding to H&E staining; **g** diffuse and (**h**) corresponding to H&E staining; **i** 100% positivity and (**j**) corresponding to H&E staining

incubated with peroxidase-labeled polymer Picture™ Max HRP polymer conjugate broad spectrum (Life Technologies^{R}, California, USA) and then with the DAB substrate chromogen (Dako^{R}, California, USA).

Technical analysis

The samples were evaluated by two researchers who determined the presence or absence of immunostaining. Staining was considered absent if no reaction was observed on the IHC sections. The quality (intensity, cell count and pattern) of Kindlin-1 and Ki-67 staining was compared using consecutive control sections, and the staining characteristics were recorded in a blinded method by two independent pathologists.

Digital images of the IHC results in three hotspots (points of maximum concentration of labeled cells) were captured [27]. For Ki-67, the pathologists performed a manual count of nuclear positivity in 500 cells at 400x magnification for each hotspot [28]. The percentage of cells expressing Ki-67 was scored as a continuous variable, from 0 to 100%. Kindlin-1 cytoplasmic qualitative staining intensity was scored as negative, focal, multifocal or diffuse. The scores of the two pathologists were compared, and any discrepancy was re-examined to achieve a consensus score. Figures 1 and 2 show some immunohistochemistry photomicrographs and their scores with each respective hematoxylin and eosin-stained section (images captured by Invitrogen EVOS FL Auto 2.0 Imaging System).

Statistical analysis

SPSS version 23.0 software for Windows (SPSS Inc., IL, USA) was used for the statistical analysis. We used Mann-Whitney tests to determine associations between Kindlin-1 and Ki-67 expression and the clinical-pathological parameters of melanoma patients, including risk factors, AJCC stage, tumor thickness, ulceration, histologic subtype and tumor location. Correlation between Kindlin-1 and Ki-67 expression was analyzed using Spearman's rank correlation. $P < 0.05$ was considered statistically significant.

Results

The patients included in the study were Caucasian, and the median age was 50.93 ± 15.31 years. Among the patients, 46.7% (14/30) were men and 53.3% (16/30) women. The characteristics of the primary tumors are shown in Table 1.

Ki-67 expression was detected in all patients (100%) with primary cutaneous melanoma, whereas Kindlin-1 expression was negative in 2 patients. Correlations between Kindlin-1 and Ki-67 expression and AJCC stages were assessed for all patients. The immunohistochemistry results are illustrated in Figs. 3 and 4.

The correlations between Kindlin-1 and Ki-67 expression and the clinicopathological features of the patients with primary melanoma are summarized in Table 2. Of the clinical risk factors, Kindlin-1 and Ki-67 expression did not correlate with skin phototype, eye color or childhood sunburn episodes ($P = 0.51$ and 0.93, $P = 0.49$ and 0.68, and $P = 0.83$ and 0.07, respectively). Among

Table 1 Clinicopathological features of primary melanoma tumor

Variable	*N*	%
Tumor Site		
Head/neck	2	6.7
Trunk	13	43.3
Upper limb	9	30
Lower limb	6	20
Histological subtype		
Superficial spreading	23	76.7
Lentigo maligna	1	3.3
Nodular	6	20
Clark's level		
I	5	16.7
II	5	16.7
III	5	16.7
IV	15	50
Breslow tickness		
Tis	5	16.7
≤ 1 mm	12	40
1.01 - 2 mm	7	23.3
2.01 - 4 mm	4	13.3
> 4 mm	2	6.7
Presence of ulceration		
Yes	5	16.7
No	25	83.3
Mitosis ratio		
Mean ± SD	2.23 ± 2.97	
Total	30	

Tis Tumor in situ, *SD* Standard deviation

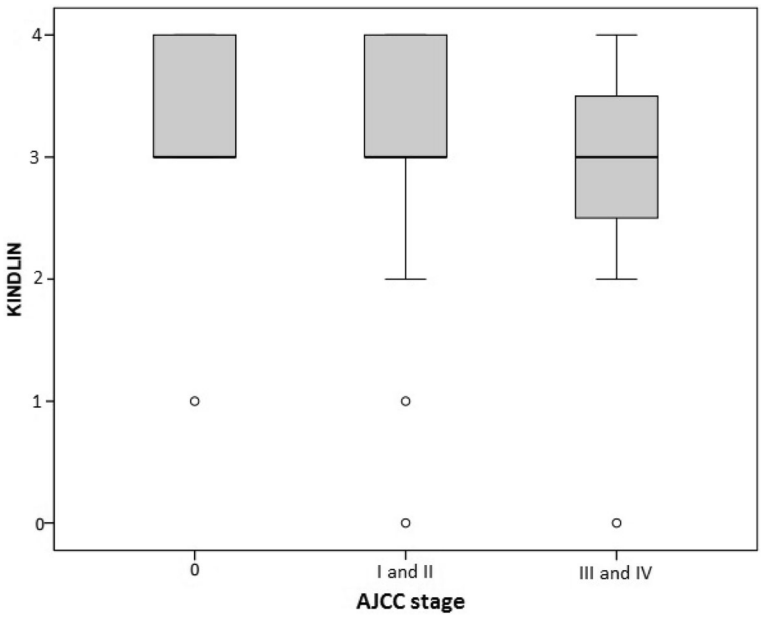

Fig. 3 Immunohistochemistry results for Kindlin-1 (intensity) and AJCC Stage (Kruskall Wallis Test; $P = 0.89$)

pathological factors, marker expression was not significantly associated with ulceration, sentinel lymph node status, Breslow thickness or mitotic rate ($P = 0.66$ and 0.51, $P = 0.31$ and 0.37, $P = 0.57$ and 0.37, and $P = 0.37$ and 0.72, respectively).

Moreover, according to Spearman's rank correlation analysis, Kindlin-1 expression did not correlate with Ki-67 expression (Table 3, $P = 0.46$), and there was also no correlation between the expression of both markers and clinical stage ($P = 0.34$ and 0.18, respectively).

Discussion

The primary objective of this study was to evaluate Kindlin-1 and Ki-67 immunostaining in a series of patients with primary melanoma. Malignant melanoma is an aggressive skin cancer due to its rapid progression, propensity to

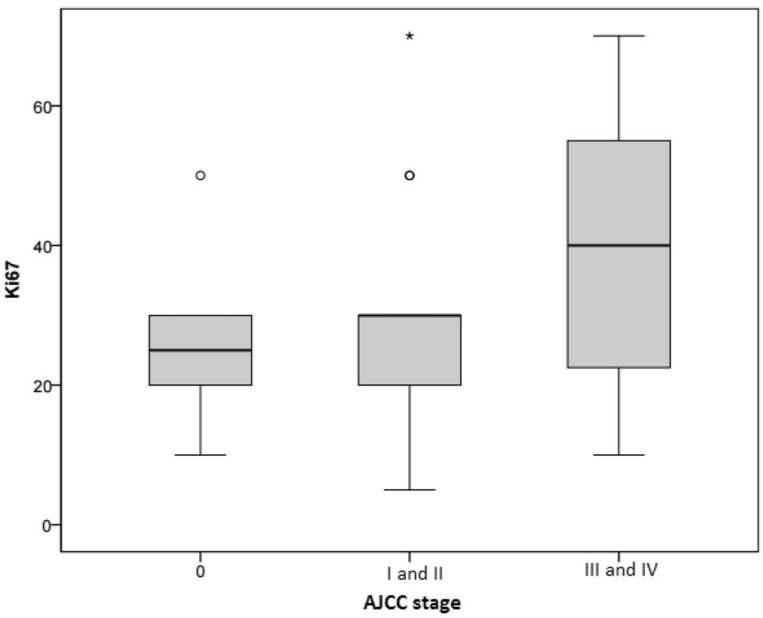

Fig. 4 Immunohistochemistry results for Ki-67 (%) and AJCC Stage (Kruskall Wallis Test; $P = 0.41$)

Table 2 Association between biomarkers level and risk and prognostic factors for melanoma (Mann-Whitney test)

Variable	N	*P*	
		KINDLIN-1	KI-67
Sunburn episodes		0.83	0.07
Yes	17		
No	13		
Skin phototype I/II		0.51	0.93
Yes	15		
No	15		
Eye color		0.49	0.68
Light	13		
Dark	17		
Presence of ulceration		0.66	0.51
Yes	5		
No	25		
SLN status		0.31	0.37
Positive	5		
Negative	22		
Breslow thickness		0.577	0.377
Mean ± SD	1.47 ± 1.85		
Mitosis rate Mean ± SD	2.23 ± 2.97	0.379	0.727
Total	30		

SLN Sentinel limph node, *SD* Standard deviation

metastasize, poor prognosis and intrinsic resistance to treatment. Because the disease is heterogeneous, tumor markers are needed to stratify these patients and to improve the accuracy of estimates of disease progression and survival. However, there is little information to date about markers for primary melanoma because there have been few studies correlating these markers with the clinicopathologic characteristics of this disease. Previous studies on tumor markers have compared dysplastic nevi, primary melanomas and melanoma metastases, finding correlations between differences in marker expression with disease progression.

Studies of tumor biomarkers are important because they facilitate disease stratification and benefit our understanding of tumor progression. Nonetheless, the most valuable biomarkers are those that indicate response to treatment [14].

A previous study showed that Kindlin-1 expression in breast tumors is associated with lung metastasis [15]. Furthermore, some evidence suggests that Kindlin-1 is a potentially important clinical mediator of lung metastasis in breast cancer and possibly other carcinomas, such as colon cancer, for which overexpression of Kindlin-1 was previously reported in a small cohort of 7 patients [16].

The Ki-67 index value is reportedly higher in malignant melanomas than in benign nevi, and this index has been correlated with prognosis in patients with melanoma. Conversely, other studies have not found a correlation between Ki-67 expression and overall survival [5, 21, 22].

There is significant heterogeneity between studies with regard to immunohistochemistry techniques, analysis methods and number of tissue samples tested. Although our study did not reveal correlations between the markers tested and risk factors or clinicopathological parameters, some previous results have shown correlations between Ki-67 and prognostic factors in cutaneous melanoma. We used a continuous variable (percentage) to measure Ki-67 staining in tissue samples, as there is no standard method in the literature for assessing Ki-67 positivity in primary melanoma.

We did not find any previous studies involving Kindlin-1 staining in primary melanomas, but there are some studies correlating Kindlin-3 and melanomas or Kindlin-1 staining and prognosis for other cancers, including skin cancer. Overall, our analysis did not show any correlations between Kindlin-1 and the clinicopathological parameters we examined.

Table 3 Correlation between stages and markers (Spearman's rank)

	Stages[a](*N* = 30)
Kindlin-1 Correlation coefficient	0.178
Sig. (2-tailed)	0.347
N Ki67 Correlation coefficient	300.247
Sig. (2-tailed)	0.188
N	30

[a] All stages (0, I, II, III and IV)

Conclusions

In our study, we did not compare primary melanoma samples and metastatic disease or dysplastic nevi, as previously conducted [10, 14, 15]. We believe that differences in marker expression may occur in each condition. Beyond this limitation, a larger cohort may find different results. We believe that additional studies are also necessary to assess whether expression of these markers correlates with clinical outcomes in patients with cutaneous melanoma.

Abbreviation

AJCC: American Joint Committee on Cancer; BSA: Bovin serum albumin; FERMT1: Fermitin family member 1; IHC: Imunohistochemistry; ISCMPA: Irmandade Santa Casa de Misericórdia de Porto Alegre; PBS: Phosphate buffered saline; UFCSPA: Universidade Federal de Ciências da Saúde de Porto Alegre; USA: United States of America

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Authors' contributions
LEHS, MAM and CGB developed the project, selected and analyzed patients data and contributed to statistical analysis. RF and PR performed and interpreted all histological examination. KCR and FR interviewed patients and performed immunohistochemistry and technical analysis. IC was a major contributor to statistical analysis and organized tables and figures. All authors read and approved the final manuscript.

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Availability of data and materials
Data of thirty patients from the files of patients diagnosed and treated in our institution (ISCMPA).

Ethics approval and consent to participate
Written informed consent was obtained from each patient according to the recommendations of the local ethics committee. This study was approved by the ethics committee of the hospital and the university where the study was developed (ISCMPA and UFCSPA, respectively).

Consent for publication
All authors are aware of this publication.

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

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