Original paper

Kindlin-3 and RASSF6 are probable biomarkers for predicting metastasis in cutaneous melanoma

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It is well known that metastasis is the most crucial factor in determining the fate of the patient. The prognosis of melanoma is very poor at the stage of metastasis. Recently, several genes and proteins, including kindlin3, dioxin receptor (AhR), RASSF6, and claudin-11, which were shown as possible prognostic biomarkers for human tumours, were described. In this study, we focused on these proteins in melanoma within a clinical setting. Forty-three primary melanomas (PMs), 17 metastatic melanomas (MMs), 15 melanocytic nevi (MN), and two melanoma cell lines were included in this retrospective study. All proteins were investigated using immunohistochemistry, and analysis was performed using a semi-quantitative immunoreactive score (IRS). The nevus group showed lower RASSF6 and AhR IRS levels than PMs. RASSF6 and kindlin-3 levels in the PMs with metastasis (MwM) and also in PMs showing lymphovascular invasion were significantly lower. The logistic regression model also proved that kindlin-3 expression was a significant independent predictor of metastasis. The current study supports the role of kindlin-3 and RASSF6 as prognostic biomarkers in melanoma. Besides the prognostic roles of these proteins, they are probably potential candidates for target-oriented therapies for melanoma metastasis blocking.

Key words: kindlin-3, AhR, RASSF6, claudin-11, melanoma, metastasis.

Introduction

Melanomas are life-threatening skin tumours, and their incidence is increasing worldwide [1]. Essentially, melanomas can be assigned to two prognostic categories: thin/early-stage melanomas usually with a good prognosis, and late-stage/metastatic melanomas (MMs) with a grave prognosis [1, 2]. Since MMs have a worse prognosis and are not easily controllable clinically, a significant number of genes and proteins have been investigated for understanding the biological mechanism of melanoma metastasis [3]. Furthermore, researchers have also focused on finding new prognostic and therapeutic targets to control metastasis.

Recently, different groups focused on several genes and proteins, including kindlin-3, dioxin receptor (AhR), RASSF6, and claudin-11, which may have prognostic importance and can suppress metastasis in human tumours [4, 5, 6, 7, 8]. In this study, kindlin-3, dioxin receptor (AhR), RASSF6, and claudin-11, recently described as predictive biomarkers in experimental studies, were studied in nevi, primary (PMs) and, MMs. Our main aim was to analyse four tumour (metastasis) suppressor proteins in primary cutaneous melanomas with positive and negative metastasis status.

Table I. Study Groups

Nevus				
n	Gender	Age	Type	Localization
15	12F/3M	Range: 14-67	Compound: 8	Head and neck: 11
		Mean: 31.4	Dermal: 7	Extremities: 3
				Body/Trunk: 1
Primary melanoma				
n	Gender	Age	Type	Localization
43	22F/21M	Range: 23-89	Acral melanoma: 18	Head and neck: 15
		Mean: 62.8	Nodular melanoma: 12	Extremities: 24
			Low CSD (superficial spreading melanoma): 9	Body/trunk: 4
			Lentigo maligna/melanoma: 4	
		pT	**M–**	**M+**
		Tis	**3**	**0**
		T1a	**5**	**0**
		T1b	**1**	**0**
		T2a	**3**	**1**
		T2b	**0**	**1**
		T3a	**4**	**4**
		T3b	**6**	**0**
		T4a	**1**	**1**
		T4b	**5**	**8**
Metastatic melanoma*				
n	Gender	Age	Localization of metastasis	Localization of primary melanoma
17	2F/15M	Range: 26-86	Skin: 1	Acral: 3
		Mean: 55.4	Internal organs: 4	Head and neck: 6
			Lymph nodes: 12	Extremities: 3
				Body/trunk: 1
				Unknown: 4

*M– primary melanoma without metastasis, M+ primary melanoma with metastasis *Unmatched with primary melanomas*

Material and methods

Study group

Forty-three PMs, 17 MMs, 15 melanocytic nevi (MN), and two established melanoma cell lines, WM-115 and WM-266-4, were included in this retrospective study. Primary melanomas were also separated into two groups, melanoma with positive metastasis status (MwM) (n = 15) and melanoma without metastasis status (MwoM) (n = 28). The data for metastasis of PM cases were acquired by sentinel node status and minimum one-year follow-up information after surgery.

All patients were Caucasians, and the detailed characteristics of the study groups are summarised in Table I. PM cases were re-evaluated, and well-known morphologic data including melanoma type (WHO-2017), tumour thickness (Breslow), anatomic level (Clark), mitosis, ulceration, in-situ component, microsatellitosis, lymphovascular invasion (LVI), and perineural invasion were also collected. Clinical data were acquired from the hospital information system.

Immunohistochemistry analysis

Immunohistochemistry technique

External and internal controls evaluated all immunohistochemically stained slides. Immunohistochemical studies were performed automatically in the Bond Max equipment (Leica Microsystems Inc., Wetzlar, Germany). Antigen retrieval steps were completed

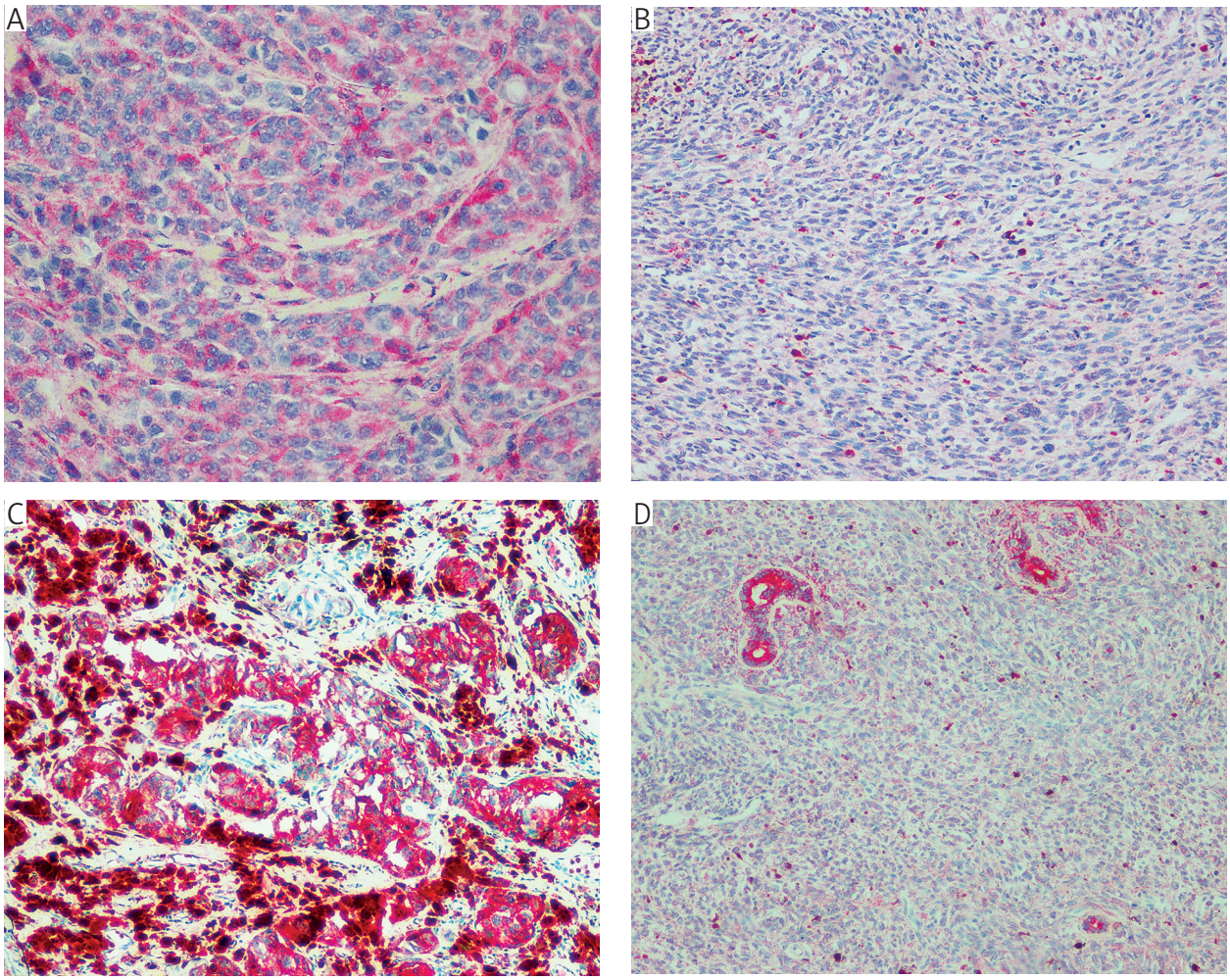


Fig. 1. Medium-strong kindlin-3 staining in a nodular melanoma case with negative metastasis status **(A),** but no staining is detected in a primary acral melanoma case with metastasis **(B)**. Two acral melanoma cases **(C; D)**, in one of the cases without metastasis, RASSF6 stains strongly (C), but the other has positive metastasis status with faint staining. (Original magnification, A, C 200×, B, D 100×)

in Bond-Epitope Retrieval Solution 1 (AR9961) for claudin-11 (oligodendrocyte specific protein) (Abcam Ab53041, 1/200, Cambridge, USA), kindlin-3 (URP2) (Abcam, Ab 173416, 1/200), RASSF6 (LS-Bio, S: B11633, 1/200), BRAF V600E (Roche VE1, prediluted, Indianapolis, USA), and Bond-Epitope Retrieval Solution 2 (AR9640) for aryl hydrocarbon receptor (AhR, dioxin receptor) antibodies (Abcam Ab84833, 1:100). Visualization was carried out with a Bond Polymer Refine Red Detection Kit (DS9390) and counterstained with haematoxylin.

Immunohistochemical analysis

Stained slides were semi-quantitatively evaluated using a specific "immunoreactive score (IRS)" described before, with minor modifications [9]. Simply, IRS is the result of multiplication of the positive cell proportion score (0-4) and the staining intensity score (0-3), which has a range of 0-12. When the examined sample stained for an immunohistochemical (IHC) marker shows heterogeneous staining, the staining intensity is scored independently, and the results are summed. The details of the analysis technique were clearly described in the literature [9].

IRS analyses were carried out using a Nikon Eclipse 80i fluorescence microscope (Nikon Europe, Amsterdam, Netherlands), and scores were calculated using Nikon NIS Elements 3.0 software (Nikon Europe, Amsterdam, Netherlands).

Cell culture

Formalin-fixed cell pellets of melanoma cell lines from the PMs and MMs of the same patient, WM-115 and WM-266-4, respectively, were available from another project of the author (OB) (Project No: 2012/59) [10]. Briefly, cell pellets cultured by classical cell culture propagation procedures with trypsinization for collection of the cells were fixed for 12 hours in neutral buffered 10% formalin. They were re-centrifuged at 2000 rpm for 5 min. Pellets

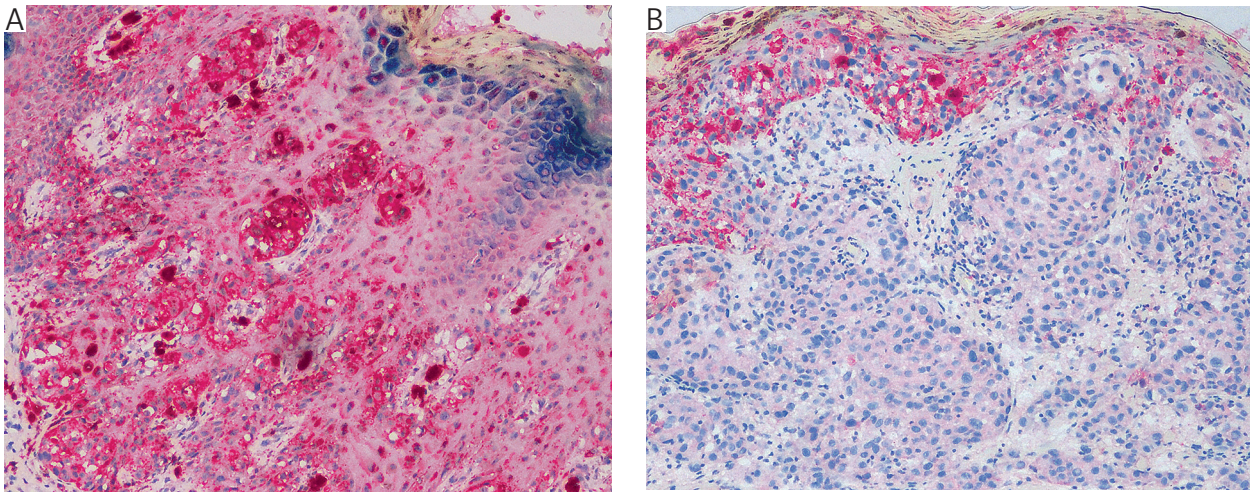


Fig. 2. Strong cytoplasmic AhR (dioxin receptor) positivity in a superficial spreading melanoma. Squamous cells show faint-medium staining with focal nuclear staining in the granular cell layer **(A)**. Similarly, an *in situ* melanoma component is highlighted but not an invasive component in another case **(B)**. (Original magnification: A 100×; B 200×)

were then wrapped in a thin filter paper, and classical tissue processing procedures and paraffin embedding as in tissue were performed.

Statistical analysis

All collected data were evaluated by the IBM PASW statistics 17.0 program (New York, USA). The IRS difference between the groups was analysed by the Mann-Whitney U non-parametric test ($p < 0.05$ accepted as significant). In the case of more than two-group comparisons, we also used Bonferroni correction. Correlations between proteins and clinicopathologic data were investigated by Spearman rho correlation tests ($r > 0.25$; $p < 0.05$ accepted as significant). A binary logistic regression model was studied to show the relationship between protein expression and metastasis status.

Ethics statement

This study was financially supported by the Scientific and Technological Research Council of Turkey (TUBITAK, grant number SBAG-116S193). The project was approved by the Ankara Numune Research and Education hospital Local Ethics Committee (24.12.2015, 706/2015).

Results

Immunohistochemical staining results

RASSF6 and kindlin-3 immunohistochemical staining positivity generally showed medium intensity in all groups with different proportions (Fig. 1). RASSF6 staining was detected in 66.6% of nevus, 90.6% of PM, and 94.1% of MM samples. On the other hand, kindlin-3 staining was observed in 86.6% of nevus, 90.4% of PM, and all MM samples.

AhR staining was detected in all of the nevi except one case, 97.6% of PMs, and 94.1% of MMs with medium and strong intensity. We could not detect nuclear positivity in the PM and MM groups, but six out of 15 nevi showed weak nuclear positivity. One interesting finding is the medium and strong AhR staining in the superficial nevus cells and the in-situ component of melanoma compared to the invasive part (Fig. 2).

Claudin-11 staining intensity was very weak in all groups of melanocytic lesions. We could not detect any positivity except weak positivity in one case in the nevus group. Weak and focal positivity was detected in 11.6% of PMs and 5.8% of MMs. Positivity was usually cytoplasmic, and rarely weak nuclear positivity was encountered in some tumours (Fig. 3).

BRAF V600E cytoplasmic staining was calibrated in the Bond Max equipment using a molecular level (*BRAF* RT-PCR) confirmed melanoma slides. BRAF V600E staining was detected in 47.6% of PMs.

Kindlin-3 showed strong positivity in WM-115 and WM-266-4 cell lines. Similarly, medium-strength RASSF6 positivity was detected in both cell lines. Claudin-11 staining was very weak in WM-115 but negative in WM-266-4. Weak AhR staining was detected in WM-115. However, it was stronger but heterogeneous in WM-266-4 (Supplement).

Comparative statistics

When we compared the nevus group with PMs and MMs, RASSF6 and IRS levels were higher in PMs ($p = 0.001$) and MMs ($p = 0.005$) than nevi. IRS levels of RASSF6 and kindlin-3 in the PMs with metastasis (MwM) ($n = 15$) showed significantly

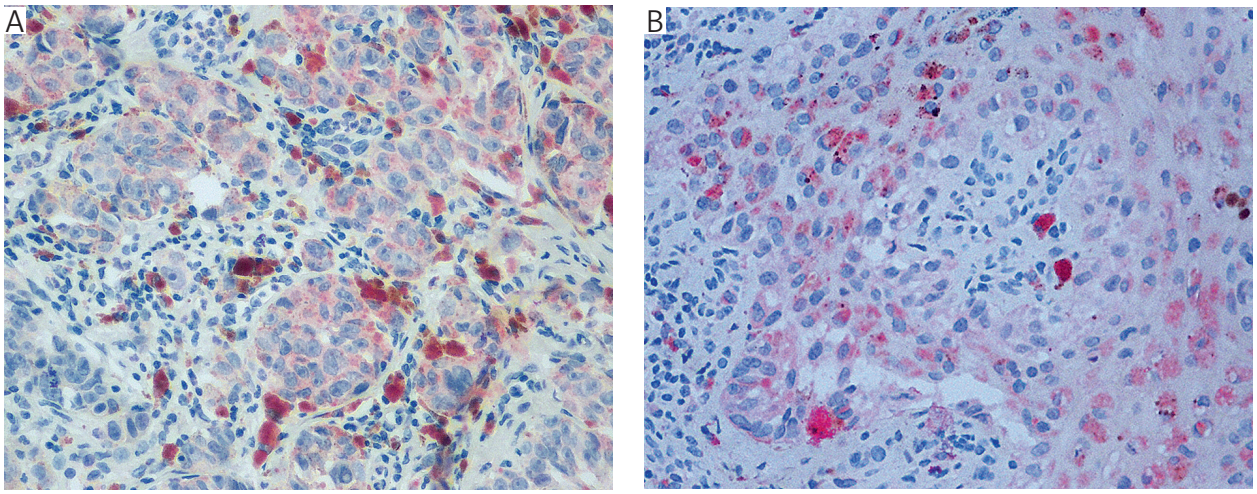


Fig. 3. Rare claudin-11 positivity in primary melanomas, but the expression strength is very low **(A; B)**. (Original magnification A, B, 200×)

lower scores than the PMs without metastasis (MwoM) (p = 0.018; p = 0.004) (Fig. 4). PMs with vascular invasions (n = 17) had a significantly lower RASSF6 score (p = 0.044) and also kindlin-3 score (p = 0.017).

We could only find a difference for claudin-11 between BRAF V600E positive and negative melanoma, with its expression being higher in mutation-positive tumours (p = 0.034).

Logistic regression

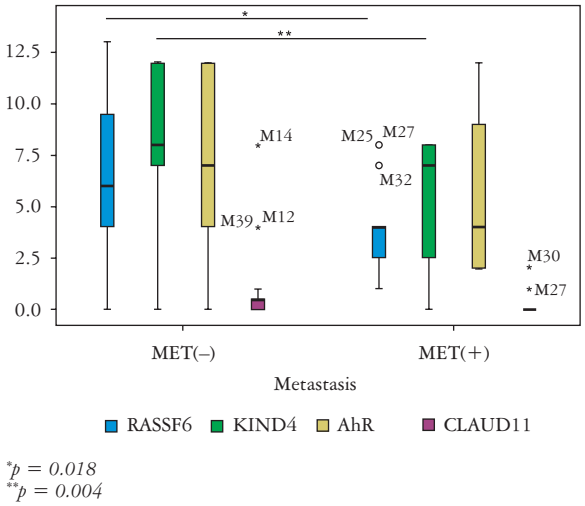
A binary logistic regression model proved kindlin-3 expression to be a significant independent predictor of metastasis (regression coefficient B = –0.239; odds ratio [OR] = 0.788; 95% confidence interval [CI] = 0.623-0.995; p = 0.045). The analysis showed that one point in the kindlin-3 staining intensity score (IS) lowers the risk of metastasis 1.26-fold (1/0.788).

Correlations

In the nevus and the MM group, there was no correlation between the studied proteins. However, significant positive correlations were detected between AhR and RASSF6 (r = 0.387, p = 0.010) and claudin-11 (r = 0.323; p = 0.035) and kindlin-3 (r = 0.422; p = 0.005); RASSF6 and claudin-11 (r = 0.532, p = 0.001) and kindlin-3 (r = 0.394; p = 0.010); claudin 11 and kindlin-3 (r = 0.425, p = 0.005) and BRAFV600E status (r = 0.340, p = 0.028), in the PM group.

Discussion

Although classical prognostic parameters such as tumour (Breslow) thickness, mitotic rate, and ulceration are well known in melanoma, there are no



*p = 0.018
**p = 0.004

Figure 4. Boxplot graph. Melanomas with positive metastasis status show significantly lower RASSF6 and kindlin-3 IRS values

validated immunohistochemical biomarkers for metastatic risk assessment despite various clinical studies [11, 12, 13]. Here, we evaluated four possible immunohistochemical markers that have shown promise as prognostic biomarkers in *in vitro* and *in vivo* studies.

Kindlin-3 (URP2, FERMT3) is one of the three members of the kindlin family which are regulators of integrin functions and have important roles in cell survival, differentiation, adhesion and migration [14, 15]. In the current study, we found that MwM and melanomas with LVI had significantly lower kindlin-3 scores. Also the logistic regression model proved kindlin-3 expression to be a significant independent predictor of metastasis. The importance of kindlin-3 in melanoma has not been well demonstrated. An immunohistochemical study showed that kindlin-3 scores were detected in melanoma rather

than nevi and normal skin [7]. Feng *et al*. reported that kindlin-3 inhibits cell migration in widely used human and mouse melanoma (B16-F10 and M10) cell lines [6]. Similar findings were demonstrated in other melanoma cell lines, including SKMEL28 and MDA-MB-231 [7].

The Ras-association domain family (RASSF) includes ten different proteins (RASSF1-to 10). RASSFs interact with different intracellular pathways, including apoptosis, cell cycle, and microtubule stabilisation [16]. RASSF6, which is one of the well-known RASSF family proteins, has significant importance in carcinogenesis. RASSF6 is considered a tumour suppressor protein because of its ability to trigger apoptosis [17, 18]. Decreased expression of RASSF6 in human malignancies is known to be of clinical importance. The decrease in RASSF6 expression is generally accepted as a worse prognostic factor in gastric [19, 20], breast [21], colorectal [22], and pancreatic [23] cancers.

In this study, although we found that RASSF6 expression was higher in PMs and MMs than MN, MwM showed lower RASSF6 scores than MwoM. A similar tendency was also found in melanomas showing lymphovascular invasion. However, we did not find any difference in RASSF6 staining between the primary and metastatic cell lines.

There are several studies regarding the importance of RASSF proteins in melanomas [4, 24, 25, 26]. It has been demonstrated that RASSF1, RASSF8, and RASSF10 have some tumour suppressor roles in melanomas [24, 25, 26]. Like in our study, Mezzanotte *et al.* reported that 73.7% of melanomas metastasising to the brain had RASSF6 promoter methylation. The authors also demonstrated *in vitro* that activation of RASSF6 in the BRAF V600E mutant cell line A375 decreased the invasion potential of the cell line [4]. As a mechanism, they proposed that RASSF6 enhanced the relationship between BRAF and MST1 proteins [4]. In this study, we did not detect any relationship between BRAF V600E mutation status and RASSF6 expression. In light of the current literature, RASSF6 expression is believed to be adversely correlated with the metastatic potential of human tumours, including melanomas. Based on the current study and the literature, it is clear that RASSF6 has a tumour suppressor function similar to kindlin-3 protein in melanoma.

Claudins are a group of proteins that have important roles in regulating tight junction formation and function [27]. Claudin-11, also called oligodendrocyte transmembrane protein, generally showed a low expression profile in this study's melanocytic neoplasms. Claudin-11 showed no expression in nevi except one case, 11.6% of PMs and 5.8% of MMs. This tendency was replicated in the cell lines. Weak claudin-11 expression was observed in the PM cell line but not in the metastatic line. This is expected in melanoma because of documented high methylation of the claudin-11 gene (*CLDN11*) and miRNA inhibition of this protein in cancer tissues in the literature. In the literature, significant methylation of the claudin-11 gene in colon carcinoma and laryngeal carcinoma has been described [28, 29]. In gastric carcinomas, Yang *et al.* demonstrated that miR-421 might promote proliferation, invasion, and metastasis by inhibiting the expression of the *CLDN11* gene [30]. Similar findings were reported in hepatocellular carcinoma due to claudin-11 inhibition by miR-99b.31 Immunohistochemical studies showed no positivity in prostate carcinomas [32]. It has also been demonstrated that claudin-11 decreases the invasiveness of bladder cancer cells [33].

In the skin, Nissinen *et al.* reported claudin-11 immunohistochemical staining in well and moderately differentiated cutaneous squamous cell carcinoma (cSCC), whereas no staining for claudin-11 was detected in poorly differentiated tumours. Furthermore, the authors also found that claudin-11 expression was specifically elevated in primary cSCC cell lines, but low or absent in metastatic cSCC cell lines and normal human keratinocyte cell lines [34]. It has been reported that claudin-11 promoter methylation was detected in nearly half of all PMs and MMs. However, methylation is lower in ordinary and dysplastic nevi [5, 35]. Though claudin-11 expression is very low, probably due to epigenetic silencing or by miRNA-based mechanisms in melanomas, the importance of this fact for melanomagenesis is not easy to explain.

One of the aims of the study was to demonstrate the differences between the studied proteins in BRAFV660E positive and negative melanomas. However, we found a difference only for claudin-11 expression between BRAFV600E positive and negative melanoma, with its expression being higher in the mutation-positive tumours. Although we could not find any data regarding the relationship between claudin-11 and *BRAF* mutations in the literature, Caruso *et al*. detected up-regulation of claudin-1 protein, another member of the claudin family, in colorectal cancer precursor lesions harbouring the BRAF V600E mutation [36].

AhR has important roles in cell and tissue homeostasis besides xenobiotic-metabolising mechanisms [37, 38]. The role of AhR in carcinogenesis is very complex. It may have a pro-oncogenic role or antitumorigenic activity related to tumour type [39]. The importance of AhR has not been clearly demonstrated in melanocytes and melanoma [40]. However, it has been proposed that AhR acts as a regulator of melanogenesis in human melanocytes [41]. Contador-Troca *et al*. focused on the role of AhR in melanoma. The authors proposed that AhR has tu-

mour suppressor activity in melanoma growth and metastasis [8, 42]. Recently, Corre *et al*. reported that AhR promotes resistance to BRAF inhibitors in melanoma [43]. In the current study, AhR expression was detected in all groups, including the cell lines used. Although we did not detect any significant correlation between Breslow thickness and AhR score ($r = –0.294$; $p = 0.055$), we observed stronger positivity in the in-situ components and the early invasive melanomas than the thick melanomas. This may show that AhR levels may be reduced when melanoma invades. However, in a recent article, Mengoni *et al*. suggested that AhR signalling might involve melanoma pathogenesis and promote tumour growth and metastasis [44]. Further studies should be performed to show the role of AhR in melanoma progression.

In conclusion, the current study suggests that kindlin-3 and RASSF6 are possible prognostic biomarkers for predicting metastasis in melanoma. One of the interesting findings from this study is the very low expression of claudin-11 in melanoma, which clearly supports the epigenetic silencing of the claudin11 gene in melanoma previously shown by several studies. Besides the prognostic biomarker roles of these proteins, they are probably candidates for target-oriented therapies for melanoma metastasis blocking.

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The authors declare no conflict of interest.

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