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ORIGINAL ARTICLE

Clinicopathological features and pituitary homeobox 1 gene expression in the progression and prognosis of cutaneous malignant melanoma

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Abstract The evidence that PITX1 (pituitary homeobox 1) is a significant tumor suppressor in human cancer remains largely circumstantial, but it clearly warrants further study as little is known about the tumor-inhibitory roles of PITX1 in cutaneous malignant melanoma. The aims of this study were to investigate *PITX1* gene expression in patients with cutaneous malignant melanoma and to evaluate its potential relevance to clinicopathological characteristics and tumor cell proliferation. Clinicopathological findings of patients with cutaneous malignant melanoma were analyzed retrospectively. PITX1 and Ki-67 expression were detected by immunohistochemistry in malignant melanoma and healthy tissue samples from each patient. Labeling indices were calculated based on *PITX1* gene and Ki-67 expression. The correlation between PITX1and Ki-67 expressions was analyzed in cutaneous malignant melanoma cases. The relationship between PITX1 expression intensity and clinicopathological characteristics was also analyzed. PITX1 expression was observed in all (100%) normal healthy skin tissue samples. In addition, PITX1 expression was found in 56 (80%) and was absent in 14 (20%) of the 70 cutaneous malignant melanoma cases. Ki-67 positive expression was only detected in the 14 (20%) PITX1-negative cases. PITX1-positive tumor cells were observed on the surface, but Ki-67 positive tumor cells were observed in deeper zones of the tumor nests. PITX1 expression was downregulated in human cutaneous malignant melanoma lesions compared with healthy

Conflicts of interest: All authors declare no conflicts of interest.

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skin tissue, but Ki-67 expression was upregulated in concordance with the progression of cutaneous malignant melanoma. PITX1 expression may be involved in tumor progression and is a potential tumor suppressor gene and prognostic marker for cutaneous malignant melanoma.

Introduction

The skin is the most common primary site of malignant melanoma, a lethal skin cancer that occurs predominantly in Caucasians [1]. Malignant melanoma originates from nevus cells and melanocytes; it is associated with poor prognosis and high mortality because of its high degree of malignancy, early blood and lymphatic metastasis, and resistance to chemotherapy and radiotherapy [2,3].

Clinicopathological features and prognostic factors for melanoma have been widely investigated within different populations [1,4,5]. The reported incidence of cutaneous malignant melanoma is high and increasing further. Cutaneous melanoma is associated with a high mortality rate, and early diagnosis and treatment are important for improving survival rates. Survival depends on tumor thickness and clinical staging on presentation [1,4]. The histological features of the primary melanoma, including tumor thickness, mitotic rate, and ulceration, are important hallmarks of melanoma prognosis and staging [6].

Pituitary homeobox 1 (PITX1/PTX1), also called Backfoot (BFT), is a *bicoid*-related homeobox transcription factor that is involved in the transcription of the pro-opiomelanocortin (*POMC*) gene [7—12]. PITX1 plays a role in the differentiation and formation of pituitary cells as well as in the development of the oral epithelium, first branchial arch, and its derivatives [13]. PITX1 is expressed throughout the developing hindlimb but not in the forelimb bud. Misexpression of PITX1 in chick wing buds causes them to develop into limbs with some morphological characteristics of hindlimbs [7—11,14].

The evidence that *PITX1* is a significant tumor suppressor gene in human cancer remains largely circumstantial (lung cancer [7], esophageal adenocarcinoma [8], osteosarcoma [9], gastric adenocarcinoma [15], colorectal adenocarcinoma [16]) but is clearly worth further study [15]. PITX1 expression and its clinical significance in cutaneous malignant melanoma remain unclear, because there are only a few studies on this subject in the literature; therefore, we focused on the expression of PITX1 in malignant melanoma tissues.

In this study, we evaluated the intensity of PITX1 expression in cutaneous malignant melanoma cases and aimed to determine its prognostic significance and its relevance to clinicopathological characteristics.

Methods

Clinicopathological patient data

Clinicopathological data were collected from patient records and their pathology reports retrospectively. Signed and written informed consent was obtained from all patients. All patients were diagnosed with cutaneous malignant melanoma in the Department of Pathology, Faculty of Medicine, Bulent Ecevit University, Zonguldak, Turkey, between January 2001 and May 2014. None of the patients had received preoperative anticancer treatment.

In total, 70 patients were evaluated, and clinical and pathological data were analyzed for each patient. Clinical characteristics included age and sex. Histological variables included histogenetic type, Clark level of invasion, Breslow tumor thickness, and the presence or absence of inflammatory infiltration (TIL) and ulceration. All tumors were classified as melanoma *in situ*, superficially spreading melanoma (SSM), lentigo malignant melanoma (LMM), nodular melanoma (NM), or acral lentiginous melanoma (ALM). Tumor thickness was considered to be a continuous variable and was categorized according to the most recently recommended breakpoints [6]: $\leq$1 mm, 1.01—2.00 mm, 2.01—4.00 mm, and $>$ 4 mm. TNM staging was assessed according to the criteria proposed by the American Joint Committee on Cancer (AJCC, 2009) [6].

Histopathological examinations were performed on 70 surgically resected cutaneous malignant melanomas and 30 healthy skin tissues. The healthy skin tissues were obtained from the margins of wide excisions taken from biopsied patient samples that were negative for dermal tumors. All specimens were fixed in 10% formalin, embedded in paraffin, and cut into 3—4-μm-thick sections. Specimens were then deparaffinized and stained with hematoxylin and eosin for histological evaluation.

Immunohistochemical examination

Sections (3—4 μm) obtained from representative tissue sample blocks were deparaffinized with xylene, rehydrated in a graded alcohol series, and placed in 0.5% hydrogen peroxide in methanol for 10 minutes to block endogenous peroxidase activity. Antigen retrieval was performed by incubation in 0.01M citrate buffer (pH 6.0) for 5 minutes in a pressure cooker. The sections were exposed to the primary antibody for 60 minutes at room temperature. The standard streptavidin—biotin—peroxidase complex method was used for PITX1 (Rabbit-polyclonal, LS-C100923; LSBio, South San Francisco, CA, USA, 1:50) and Ki-67 (Rabbit-monoclonal, RM-9106-S; Lab-Vision, Fremont, CA, USA, 1:100). 3,3′-Diaminobenzidine tetrahydrochloride was used as the chromogen. The section was then counterstained with hematoxylin, rinsed, and mounted. The sections were evaluated in a single-blinded fashion.

Immunohistochemical examinations of the specimens were based on a scoring method described by Osaki et al. [11]. At least 500 melanoma cells in the tumor area were

scored, and the percentage of cells showing positive nuclear staining was designated as the labeling index. The immunoreactivity against PITX1 protein and Ki-67 were classified into two groups: negative, when $< 10\%$ of tumor cells showed positive signals, versus positive, when at least 10% of tumor cells showed positive signals.

Statistical analysis

Statistical analyses were performed using SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). Distribution of the data was determined using Shapiro–Wilk test. Continuous variables were expressed as median (minimum – maximum) or means ± standard deviation, and categorical variables as frequencies and percentages. Continuous variables were compared using the Mann–Whitney *U* test, and categorical variables were compared using the Chi-square test. A p value of < 0.05 was considered statistically significant for all tests.

Results

PITX1 and Ki-67 expression were evaluated in 30 healthy skin samples and 70 cutaneous malignant melanoma samples. A diagnosis of malignant melanoma was made based on hematoxylin and eosin sections and HMB45 expression (Figure 1).

The correlations between immunohistochemical PITX1 expression and clinicopathological features in the 70 cutaneous malignant melanoma samples are shown in Table 1. PITX1 expression was positive in 56 (80%) and negative in 14 (20%) of the tested melanoma cases. Data from 70 patients (32 women and 38 men), with a median age of 61.50 years (range, 16–90 years), were analyzed.

There were no significant differences in age ($p = 0.336$) or sex ($p = 0.589$) between the PITX1-positive and PITX1-negative groups. However, there were significant differences in ulceration ($p < 0.001$), TIL ($p = 0.005$), thickness ($p < 0.001$), Clark's level ($p = 0.003$), histologic subtype ($p = 0.001$), and stage ($p < 0.001$) between the two groups.

The median (min – max) tumor thickness was 3.0 mm (range, 1.0–14.0 mm) in the PITX1-positive cases and 7.0 mm (range, 5.0–15.0 mm) in the PITX1-negative cases. The median tumor thickness was significantly higher in the PITX1-negative cases compared with the PITX1-positive cases ($p < 0.001$).

In this study, the most common histologic subtype was NM (35 cases), which showed the most frequent PITX1 negativity ($n = 13$). The remaining PITX1-negative case ($n = 1$) was the ALM histologic subtype. There were significant differences between NM and other histologic subtypes with respect to ulceration ($p = 0.042$), TIL ($p = 0.028$), thickness ($p < 0.001$), Clark's level ($p < 0.001$), stage ($p < 0.001$), and PITX1 expression ($p = 0.001$).

PITX1-positive (80%) and Ki-67-positive (20%) cases were analyzed, and their statistically significant correlations with stage are summarized in Table 2.

All five melanoma *in situ* cases, which were Stage 0, were classified as PITX1-positive. All Stage 0 cases (5 of 5, 100%), all Stage I cases (10 of 10, 100%), 40 of 48 Stage II cases (83.3%), one of four Stage III cases (25%), and no Stage IV cases (0 of 3, 0%) were PITX1-positive, indicating that PITX1 expression is downregulated during malignant melanoma progression. The number of Ki-67-positive cases was eight of 48 (16.7%) Stage II patients, three of four (75%) Stage III patients, and three of three (100%) Stage IV patients, indicating that Ki-67 expression is upregulated during cutaneous malignant melanoma progression.

As shown in Figure 2, PITX1-positive cells and Ki-67-positive cells present in healthy skin and cutaneous malignant melanoma samples demonstrated that PITX1 and Ki-67 are localized in the nucleus. In all 30 healthy skin samples, PITX1 was found in all epidermal layers (Figure 2A). The data indicate that PITX1 is expressed in melanocytes of healthy skin. Ki-67 immunoreaction was positive only in epidermal basal cells (Figure 2B). Ki-67 expression was negative (Figure 2D) in the PITX1-positive malignant melanoma cases (Figure 2C) and was positive (Figure 2F) in the PITX1-negative cases (Figure 2E).

PITX1-positive tumor cells were observed on the surface but were rare in deeper zones of the tumor nests in cutaneous malignant melanoma cases (Figure 3A), suggesting that PITX1 expression is suppressed in the invasive tumor front. By contrast, Ki-67-positive tumor cells were observed in deeper zones of the tumor nests (Figure 3B), suggesting that Ki-67 expression is highest in the invasive tumor front. These results indicate that the proliferative activity of melanoma cells is inversely associated with PITX1 expression.

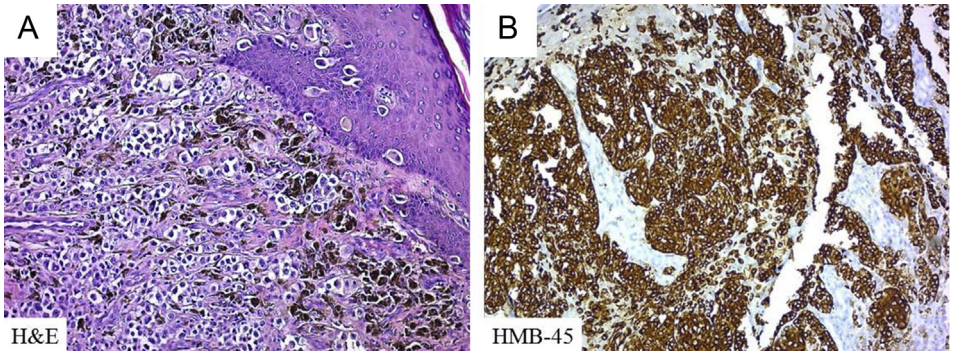

Figure 1. Histopathological appearance of cutaneous malignant melanoma in (A) hematoxylin and eosin (H&E, ×200) and (B) immunohistochemical HMB45 positivity. B-SA = 3,3′-diaminobenzidine tetrahydrochloride (×100).

Table 1 Clinicopathological and immunohistochemical features of cutaneous malign melanoma.

	PITX1		*p*
	Positive ($n = 56$)	Negative ($n = 14$)	
Age (y)			
Minimum	16	38	0.336
Maximum	83	90	
Median, range	60.0	66.5	
Sex			
Male ($n = 38$)	29 (51.8)	9 (64.3)	0.589
Female ($n = 32$)	27 (48.2)	5 (35.7)	
Ulceration			
Yes ($n = 32$)	18 (32.1)	14 (100)	<0.001
No ($n = 38$)	38 (67.9)	0 (0)	
Histologic subtype			
In situ ($n = 5$)	5 (100)	0 (0)	0.001
SSM ($n = 22$)	22 (100)	0 (0)	
LMM ($n = 1$)	1(100)	0 (0)	
ALM ($n = 7$)	6 (85.7)	1 (14.3)	
NM ($n = 35$)	22 (62.9)	13 (37.1%)	
Inflammatory infiltrate (TIL)			
Absent ($n = 31$)	30 (53.6)	1 (7.1)	0.005
Present ($n = 39$)	26 (46.4)	13 (92.9)	
Thickness (mm)			
≤1 ($n = 10$)	10 (17.9)	0 (0)	<0.001
1.01−2 ($n = 13$)	13 (23.2)	0 (0)	
2.01−4 ($n = 20$)	20 (35.7)	0 (0)	
>4 ($n = 27$)	13 (23.2)	14 (100)	
Clark's level			
0 ($n = 5$)	5 (8.9)	0 (0)	0.003
I ($n = 1$)	1 (1.8)	0 (0)	
II ($n = 8$)	8 (14.3)	0 (0)	
III ($n = 9$)	9 (16.1)	0 (0)	
IV ($n = 31$)	26 (46.4)	5 (35.7)	
V ($n = 16$)	7 (12.5)	9 (64.3)	
Stage			
0 ($n = 5$)	5 (8.9)	0 (0)	<0.001
I ($n = 10$)	10 (17.9)	0 (0)	
II ($n = 48$)	40 (71.4)	8 (57.1)	
III ($n = 4$)	1 (1.8)	3 (21.4)	
IV ($n = 3$)	0 (0)	3 (21.4)	

Data are presented as *n* (%).
ALM = acral lentiginous melanoma; *In situ* = melanoma *in situ*; LMM = lentigo malignant melanoma; NM = nodular melanoma; PITX1 = pituitary homeobox 1; SSM = superficially spreading melanoma.

Table 2 PITX1 and Ki-67 expression positivity and distribution according to stage.

Stage	Positive		*p*
	PITX1 ($n = 56$)	Ki-67 ($n = 14$)	
0 ($n = 5$)	5 (100)	0 (0)	<0.001
I ($n = 10$)	10 (100)	0 (0)	
II ($n = 48$)	40 (83.3)	8 (16.7)	
III ($n = 4$)	1 (25)	3 (75)	
IV ($n = 3$)	0 (0)	3 (100)	

Data are presented as *n* (%).
PITX1 = pituitary homeobox 1.

Discussion

In this study, we demonstrated that PITX1 expression is downregulated in cutaneous malignant melanoma tissues, compared with healthy skin samples. In addition, PITX1 downregulation was associated with clinical stage progression in malignant melanoma patients. Increased cell proliferation has been implicated in the progression of cutaneous malignant melanoma; therefore, we examined whether reduced PITX1 expression was correlated with Ki-67 expression in the malignant melanoma cases. The prognosis of cutaneous malignant melanoma depends on the stage. The 2009 AJCC staging criteria [6] for cutaneous melanoma were based on histological features such as thickness of the tumor, level of invasion, presence of ulceration/mitoses, and presence of regional and distant metastases. When we evaluated the relationship between PITX1 expression and stage, PITX1 expression was significantly reduced in advanced stage disease compared with early stage disease. These data indicate that PITX1 may be involved in tumor progression and is a potential tumor suppressor and prognostic marker for cutaneous malignant melanoma.

Homeodomain transcription factors play fundamental roles in directing cellular proliferation, differentiation, and cell fate [17]. A common feature of homeobox genes is the presence of a highly conserved 61-amino acid motif, the homeodomain. Several homeobox genes have been implicated in neoplastic development [7,11]. For example, PITX1 expression is reduced in Barrett's esophagus and is significantly reduced during the progression to Barrett's-associated adenocarcinoma [8,11]. This phenomenon is also observed in lung [7], gastric [15], and colon cancer cells [16] as well as in prostate [18] and bladder cancers, but not in the respective healthy tissues [11,19,20]. Osaki et al. [11] demonstrated that PITX1 expression is involved in the tumorigenesis and/or progression of cutaneous malignant melanoma.

Osaki et al. [11] reported that 19 (47.5%) out of 40 cases with primary melanoma were negative for PITX1 expression and found that PITX1 expression was significantly reduced during the advanced stage cancers. In this study, 14 (20%) of 70 melanoma samples were negative for PITX1 expression, and PITX1 expression was significantly reduced in advanced stage cancers. These results are therefore consistent with those of Osaki et al. [11]. The low rate of PITX1 negativity in this study is likely attributable to the small number of advanced-stage cancer samples ($n = 7$) included. Osaki et al. [11] also reported that the mean tumor thickness in the PITX1-negative cases (7.11 ± 10.3 mm) was significantly higher than that in the PITX1-positive cases (1.90 ± 3.19 mm). The present study also found that PITX1-negative tumors were thicker; however, tumor thickness was > 4 mm in all PITX1-negative cases. Tumor thickness has consistently been reported to be the most important independent prognostic variable in melanoma [21,22].

Reduced PITX1 expression in cutaneous malignant melanoma was significantly associated with several

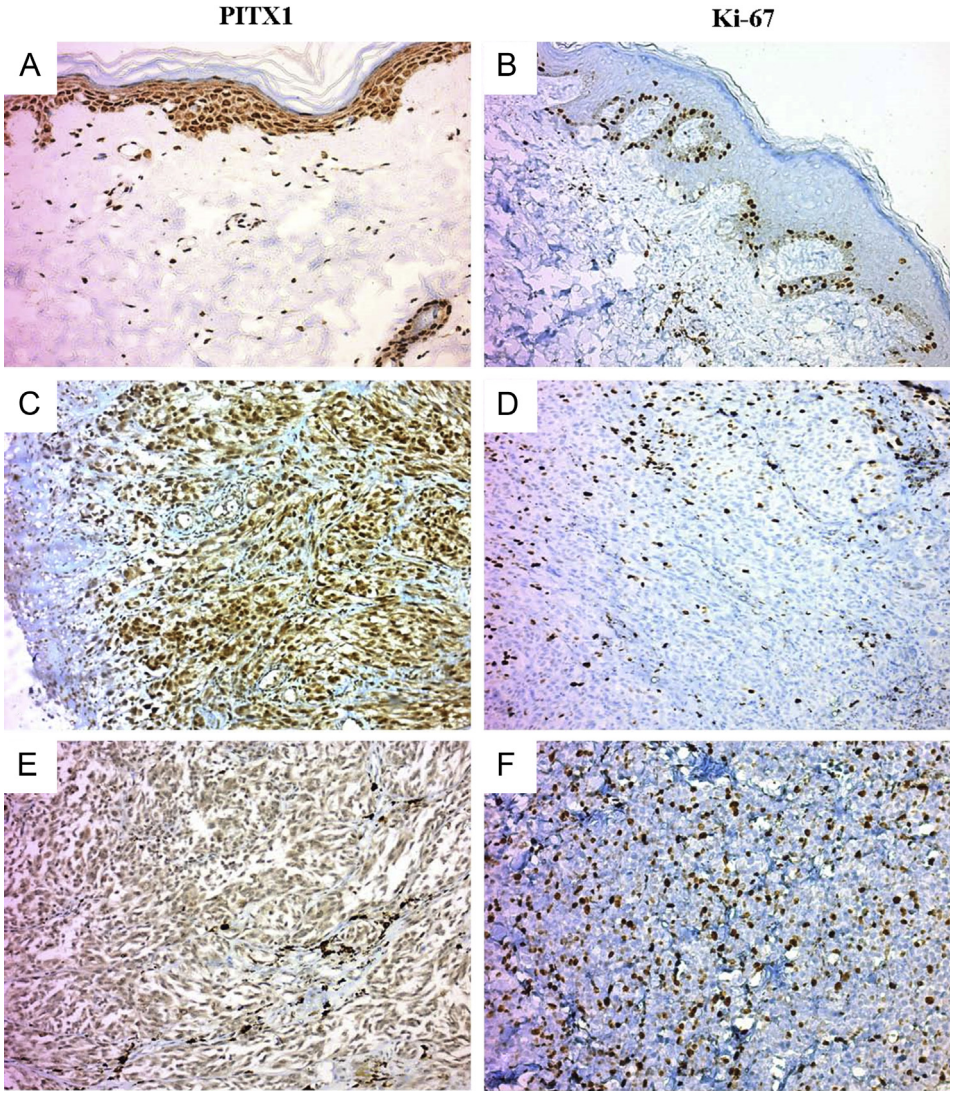

Figure 2. PITX1 and Ki-67 expression in healthy skin and cutaneous malignant melanoma samples. (A) PITX1 expression in all layers of the epidermis and skin appendages of healthy skin. (B) Ki-67 expression in the basal layer of healthy skin epidermis. (C) PITX1 positivity in cutaneous malignant melanoma (at least 10% of tumor cells showing positive staining in the nuclei). (D) Ki-67 negativity in cutaneous malignant melanoma (< 10% of the labeling index). (E) PITX1 negativity in cutaneous malignant melanoma (< 10% of tumor cells showing positive signals). (F) Ki-67 positivity in cutaneous malignant melanoma (> 10% of the labeling index). B-SA = 3,3′-diaminobenzidine tetrahydrochloride (A, B, ×200; C—F ×100); PITX1 = pituitary homeobox 1.

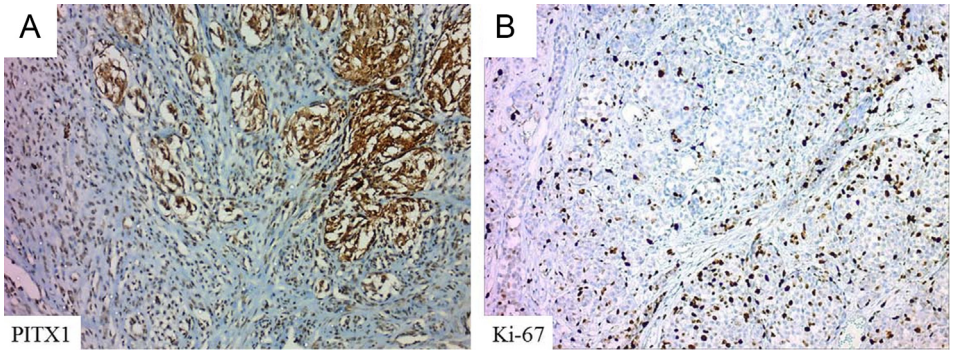

Figure 3. (A) PITX1-positive tumor cells on the surface of the tumor nests and (B) Ki-67-positive tumor cells in the deeper zone of the tumor nests in cutaneous malignant melanoma. B-SA = 3,3′-diaminobenzidine tetrahydrochloride (×100); PITX1 = pituitary homeobox 1.

clinicopathological factors including tumor thickness, metastatic rate, and clinical stage, suggesting that the loss of PITX1 expression might be a potential biomarker for cutaneous malignant melanoma malignancy [11]. Similar results were found in the present study.

The most common histologic subtype reported in the literature is the SSM [4,5,23] subtype, but in this study, the most common histologic subtype was NM. In concordance with our study, Pailoor et al. [1] also found that the most common histologic subtype was NM ($n = 22$) among 24 cutaneous malignant melanoma cases, whereas Luk et al. [4] reported that the most common histologic subtype was ALM (50.8%) among 63 cases.

The intermediate filament nestin, a neural stem-cell marker, is expressed more strongly in melanomas compared with benign melanocytic lesions, and increased nestin expression is associated with aggressive NM [24]. Increased expression of dysadherin, a novel cell membrane glycoprotein, is a significant indicator of poor prognosis in patients with cutaneous malignant melanoma and is significantly correlated with nodular subtype [25]. In the abovementioned studies, increased nestin and dysadherin expression was associated with aggressive NM and poor prognosis. In this study, a relationship between PITX1 negativity and histologic type was found; one of 14 PITX1-negative cases was the ALM and the remaining the NM ($n = 13$) histologic subtype. Of the four major histologic types, previous studies have shown that SSM and LMM have better patient survival rates compared with NM or ALM [21].

Cutaneous malignant melanoma is the most aggressive skin cancer, and its incidence is increasing worldwide [4]. Although early cutaneous malignant melanoma is curable by surgical excision, metastases develop in up to 20% of cases because of a high potential for invasion and rapid spread to other organs. Cutaneous malignant melanoma has the highest mortality rates of any cancer, particularly in advanced cases. Although considerable improvements have been made in diagnostic techniques, surgical treatment, chemotherapy, and radiotherapy in recent decades, patients with advanced cutaneous malignant melanoma still face a poor prognosis. Therefore, identification of molecular markers to predict the potential for tumor progression and disease prognosis is needed [11].

Our focus of interest in this study was the relationship between tumor PITX1 expression and/or progression of cutaneous malignant melanoma. As there is no clear link between the reported expression patterns or functions of PITX1 in cutaneous melanocytes, PITX1 expression in human cutaneous malignant melanoma was investigated in this study. The low expression of PITX1 suggested that it has a tumor suppressive function, which led us to examine the relationship between PITX1 expression and tumor progression [11].

PITX1 is a novel human telomerase reverse transcriptase (*h*TERT) suppressor gene [17,26,27]. TERT is the principal component controlling telomerase activity that facilitates cellular immortalization. PITX1 represses *h*TERT transcription through direct binding to the TERT promoter and eventually leads to inhibition of telomerase activity and proliferation [26,27]. PITX1 suppresses tumorigenicity by downregulating the RAS pathway through RASAL1, a member of the RAS-GAP family of genes (GTP-activating factor-negative regulators of RAS) [19,26]. However, the underlying mechanisms that are involved in the regulation of PITX1 remain unknown. Here, Ohira et al. [26] report that the microRNA-19b (miR-19b) regulates *h*TERT expression and cell proliferation through inhibition of PITX1. Compared with normal melanocyte cells, miR-19b expression was higher in most melanoma cells and was accompanied by downregulation of PITX1. Moreover, overexpression of miR-19b inhibited PITX1 mRNA translation through a miR-19b binding site within the 3′UTR of the PITX1 mRNA. Ohira et al.'s [26] combined findings indicate the participation of miR-19b as a novel upstream effector of *h*TERT transcription via direct targeting of PITX1 [26].

Activation of the RAS/MAPK pathway contributes to the tumorigenesis and progression of cutaneous malignant melanoma, and inhibition of the pathway could suppress the proliferation of human melanoma cells [11,28].

Consistent with our study, Osaki et al. [11] showed that the Ki-67 labeling index, indicative of proliferative activity, was significantly higher in PITX1-negative tumor areas compared with PITX1-positive tumor areas. Therefore, they suggested that loss of PITX1 expression results in activation of the RAS/MAPK pathway via downregulation of RASAL1, leading to hyperproliferation of melanoma cells [11]. Our study supports this conclusion.

The purpose of this study was to reveal the relationship between PITX1 and cutaneous malignant melanoma. Downregulation of PITX1 in melanoma cells may be involved in the progression of cutaneous malignant melanoma; thus, PITX1 represents a candidate tumor suppressor gene. Further studies should examine potential mechanisms for modulating PITX1 expression during the progression of cutaneous malignant melanoma.

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