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Original Article

Increased CDK4 protein expression predicts a poor prognosis in mucosal melanoma associated with the p16^{INK4a}-CDK4-pRb pathway

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Abstract: Mucosal melanoma (MM) occurs in non-cutaneous mucosal sites, e.g., the head and neck or the lower genital tract; it is a rare and aggressive neoplasm with a poor prognosis. To date, few prognostic markers of MM have been well-defined. The aim of this study is to clarify the prognostic value of the cell-cycle regulatory proteins (CDK4, pRb and CyclinD1, p16) which are associated with the p16^{INK4a}-CDK4-pRb pathway in MM. A total of 54 MM samples were obtained from biopsy specimens, and the expressions of the cell-cycle regulatory proteins (CDK4, pRb and CyclinD1, p16) were assessed by immunohistochemistry. A Mantel-Cox regression analysis was performed to investigate the association of these proteins with the overall survival of MM patients. Increased CDK4 expression was significantly associated with reduced survival at three years (P = 0.022). Increased CDK4 protein expression may be a helpful prognostic indicator for the management of these patients who infiltrate into the p16^{INK4a}-CDK4-pRb pathway. In addition, we found that those patients with low expression of CDK4 were significantly older (P < 0.05) compared to the patients with high expression of CDK4.

Keywords: CDK4 protein, mucosal melanoma, prognosis

Introduction

Mucosal melanoma (MM) may originate in non-cutaneous mucosal sites, including the head and neck or the lower genital tract. The biological behavior of these tumors is not only worse than their cutaneous counterparts, but there is an increased incidence of metastasis after removal. It is often diagnosed at a later stage because of anatomical considerations. The prognosis of melanoma is determined by the primary tumor thickness. Ulceration, mitosis, lymphatic spread, and distant metastasis are closely related to the prognosis, and the structure of MM is different from cutaneous melanoma. In general, MM is mostly polypoid, nodular, and irregular. It is dark, with ulcers and erosions on the surface mucosa, which is different from cutaneous melanoma. MM lacks a codified clinicopathologic staging system for the former lesions [1], and we could identify no microscopic features of MM that are related to its prognosis. MM-related prognostic immunomarkers have a practical significance. At present, the cell proliferation index marker Ki-67 is recognized as important in MM.

Derailments of the control mechanisms in the G1/S phase of the cell cycle play a fundamental role in the initiation and progression of some cancers [2]. Genetic aberrations of cell-cycle regulators, such as CDK4, CCND1, and CDKN2A, are common in melanoma and are potentially susceptible to CDK4/6 inhibition, with poorer outcomes in the context of the inhibition of the MAPK pathway [3].

The key roles of cyclin-dependent kinases (CDK) and D-type cyclins (CCND) in cell cycle progression from the G1 to the S phase were discovered more than 20 years ago [4]. CDK 4/6 activity is regulated by the INK4 family of proteins. Among them, p16^{INK4a} appears to be the most relevant, in terms of tumor suppression activity [5]. Alterations of the expression of the p16^{INK4a}-CDK4-pRb pathway components or their direct regulators may result in cell cycle progression and cell proliferation representing a potential mechanism for tumorigenesis.

The solid tumors for which the $p16^{INK4a}$-CDK4-pRb pathway is more frequently deregulated through direct genetic, epigenetic or transcriptional modifications are melanoma, and other malignant tumors [6]. However, only a few studies have addressed the issue of the simultaneously occurring abnormalities of the $p16^{INK4a}$-CDK4-pRb pathway components in melanoma.

Materials and methods

Mucosal melanoma specimens were collected from various anatomical locations (including from the nasal cavity, the esophagus, the gastrointestinal tract, the orbit, vagina, urethra, and the lungs) from 2008 to 2017. A retrospective follow-up was systematically performed, centering on the overall survival time with other clinical information (name, age, gender, lesion location, and cellular morphology, etc.).

MM was diagnosed by two pathologists mainly on the basis of histological examination of the hematoxylin-eosin stained tissue sections according to the WHO criteria. The histologic examination revealed intratumoral heterogeneity, including epithelioid, spindle-shaped, and small-cell cytomorphology. MM was diagnosed directly when the tumor cells were melanin-rich and positive for S0X10, HMB45, MelanA, S100, and Ki67 as previously described. Immunohistochemical staining was used for confirmation. The samples were primary tumors acquired from excisional surgery. Data regarding the patients' ages, genders, and lesion sites were recorded, and paraffin blocks of formalin-fixed samples were obtained. The protocol of this research was approved by the Wenzhou Medical University ethics committee.

Immunohistochemical analysis

For the immunohistochemical analysis, 3.5-micrometer sections were cut and placed on poly-L-lysine slides, warmed up to 70°C, deparaffinized with xylene, and dehydrated with various degrees of alcohol. For antigen retrieval, submersion in a citrate buffer (pH = 7.4-7.6, M = 0.01) and heating under pressure in a microwave oven were performed. To block endogenous peroxidase, the sections were placed in hydrogen peroxide.

The immunohistochemical staining was performed using the CDK4, pRb, CyclinD1, and the p16 primary antibodies (ZSGB-BIO, China) in the 54 samples according to the manufacturer's instructions at room temperature for 30 min. The slides were then cleaned in a phosphate saline buffer and incubated with a streptavidin-biotin peroxidase detection kit (DAKO), and once more, they were washed in a phosphate saline buffer followed by flooding with diaminobenzidine chromogen (DAB). Finally, Mayer's hematoxylin solution was used as a counterstain. Negative controls were achieved by staining sections from patients with nasal polyposis or mucositis.

All slides were reviewed by two pathologists under a double-headed lighted microscope, and disagreements were resolved by consensus. With the nuclear markers CyclinD1, pRb, no confusion occurred in interpreting the results of melanin deposition in cells, as melanin is cytoplasmic and causes no problem with nuclear markers. However, despite the cytoplasmic/nuclear staining of CDK4 and p16, the clearly stained nuclei in the melanoma cells were diagnostic. Suspicious samples were compared with the hematoxylin and eosin sections. In these cases, it was easy to differentiate the positively stained cells from cells with melanin deposition.

The staining intensity was scored using the following scale: no staining (0), weak (1), moderate (2), and strong (3). The immunoreactive score (IRS) was then used to determine the staining level by staining intensity, and this IRS was used to grade the protein expression. IRS greater than or equal to 2 was considered a high expression, but IRS less than or equal to 1 was considered a low expression.

Statistical analysis

The statistical analysis was done using the R program. The Kaplan-Meier method was utilized to graph the survival curves. A log-rank test was used to analyze the significant differences of the Kaplan-Meier curves. The overall risk of death was estimated using hazard ratios (HRs), and a 95% confidence interval (CI) was determined using the Cox regression model. An χ^2 test was utilized to determine the differences in the clinicopathologic features between pairs of groups. In all the analyses, a P-value ≤ 0.05 was considered statistically significant.

Table 1. Characteristics of the patients

Characteristics	Number of patients (%)		CDK4		
			Low#	High##	*P* value*
Total patients		54	35 (64.8)	19 (35.2)	
Age (year), median (range)		73 (31-96)			
	≤ 73	26 (48.1)	14 (53.8)	12 (46.2)	
	> 73	28 (51.9)	21 (75.0)	7 (25.0)	0.003*
Gender, N (%)					
	Male	29 (53.7)	17 (58.6)	12 (41.4)	
	Female	25 (46.3)	18 (72.0)	7 (28.0)	0.477
Anatomic sites					
	Head and neck	44 (81.5)	28 (63.6)	16 (36.4)	
	Gastrointestinal	6 (11.1)	5 (83.3)	1 (16.7)	
	Genitourinary	3 (5.5)	2 (66.7)	1 (33.3)	
	Lung	1 (1.9)	1 (100.0)	0 (0.0)	0.319
Survival, N (%)					
Three-year survival	Alive	18 (33.3)	13 (72.2)	5 (27.8)	
	Deceased	36 (66.7)	22 (61.1)	14 (38.9)	0.022*
Cellular morphology					
	Epithelioid cells	47 (87.0)	29 (61.7)	18 (38.3)	0.182
	Spindle cells	7 (13.0)	6 (85.7)	1 (14.3)	

#Low indicates the intensity score of CDK4 expression as no staining or weak staining. ##High indicates the intensity score of CDK4 expression as moderate or strong staining. **P*-value ≤ 0.05 was considered statistically significant.

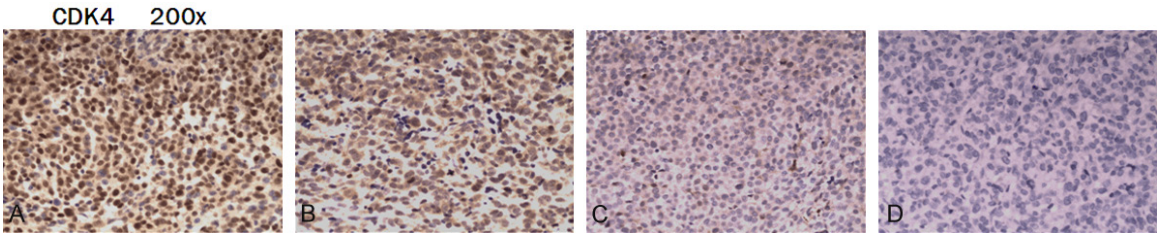

Figure 1. The staining intensity of CDK4 which was scored using the following scale from left to right: (A) strong, (B) moderate, (C) weak, and (D) no staining.

Results

The MM patients consisted of (25/54, 46.3%) female and (29/54, 53.7%) male patients. Overall, the median age of the patients was 73 years (range 31 to 96). The clinical data of the 54 MM patients are summarized in **Table 1**. The primary lesions were distributed in the head and neck (44/54, 81.5%), the gastrointestinal system (6/54, 11.1%), the genitourinary systems (3/54, 5.5%) and the lungs (1/54, 1.9%) respectively. The head and neck MM patients consisted of 37 cases in the nasal cavity, 2 cases in the paranasal sinuses, 3 cases in the ocular orbit, and 2 cases in the oral cavity. Neither a high expression nor a low expression of CDK4 significantly correlated with the clinical or pathological characteristics (gender, anatomic sites, or cellular morphology) (summarized in **Table 1**). Interestingly, patients with low expressions of CDK4 were significantly older ($P < 0.05$) compared to the patients with high expression of CDK4.

According to our findings, the results for protein high expression of CDK4, pRb, CyclinD1, and p16 were obtained for 8, 13, 19, and 30 cases, respectively. For CDK4, strong and moderate staining occurred in 35.2% (19/54), and weak in 57.4% (31/54), and no staining in 7.4% (4/54) of tumors was observed (**Figure 1**). For pRb, strong and moderate staining occurred in 14.8% (8/54), and weak in 33.3% (18/54), and no staining in 51.9% (28/54) of tumors was

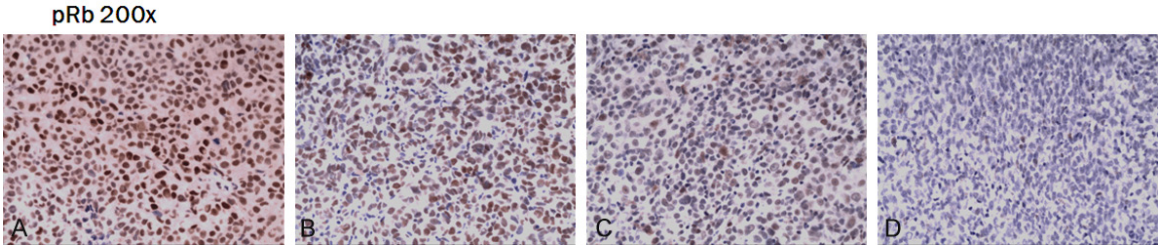

Figure 2. The staining intensity of pRb which was scored using the following scale from left to right: (A) strong, (B) moderate, (C) weak, and (D) no staining.

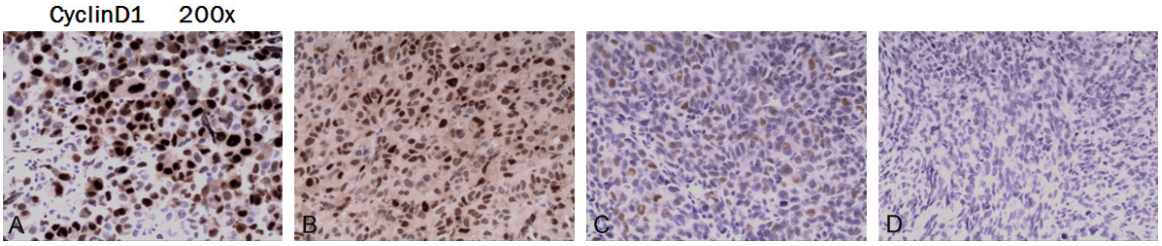

Figure 3. The staining intensity of CyclinD1 which was scored using the following scale from left to right: (A) strong, (B) moderate, (C) weak, and (D) no staining.

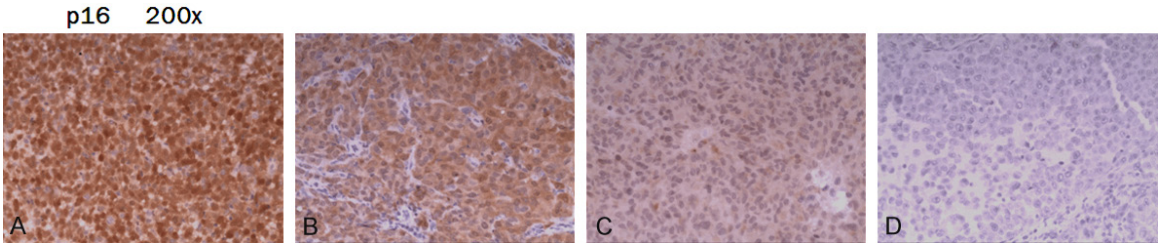

Figure 4. The staining intensity of p16 which was scored using the following scale from left to right: (A) strong, (B) moderate, (C) weak, and (D) no staining.

observed (**Figure 2**). For CyclinD1, strong and moderate staining occurred in 24.1% (13/54), and weak in 40.7% (22/54), and no staining in 35.2% (19/54) of tumors was observed (**Figure 3**). For p16, strong and moderate staining occurred in 55.6% (30/54), and weak in 18.5% (10/54), and no staining in 33.3% (18/54) of tumors was observed (**Figure 4**). Patients with high expression of CDK4 had unfavorable three year survival rates (**Figure 5**) ($P < 0.05$), but there was no statistical significance between the patients' survival and the different expression of pRb, CyclinD1, p16 (**Figures 6-8**).

Discussion

A study of a large cohort of patients with primary invasive melanoma shows that gene alterations of the CDK4 pathway are common [6]. Cyclin-dependent kinase 4 (CDK4) is a serine/threonine kinase that is a central regulator of the G1-S transition of the cell cycle. p16 is directly involved not only in controlling tumor growth, but also in regulating the normal cell cycle in a negative feedback manner. p16 molecules bind cyclin-dependent kinase 4 (CDK4) to suppress the activity of CDK4 and CDK6-cyclinD kinase, preventing the pRb protein and separating the E2F from codifying a family of transcription factors (TF) in higher eukaryotes. As a consequence, the non-phosphorylated Rb leads to blocking the cell cycle and the suppression of cell proliferation [7]. Subsequent phosphorylation of Rb(pRb) by the CDK2/cyclinE complex diminishes its ability to repress RNA polymerase I and III thus impacting protein synthesis, and represseses gene transcription

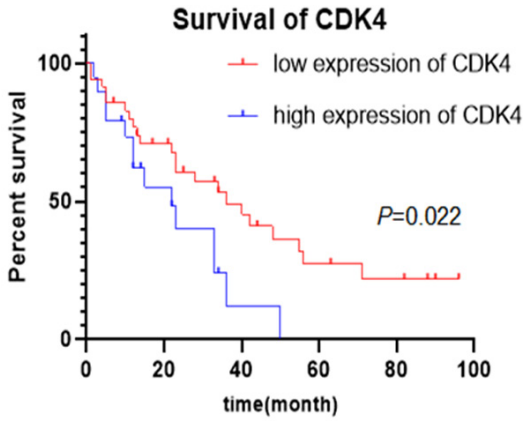

Figure 5. Kaplan-Meier survival curves of the different expression of CDK4.

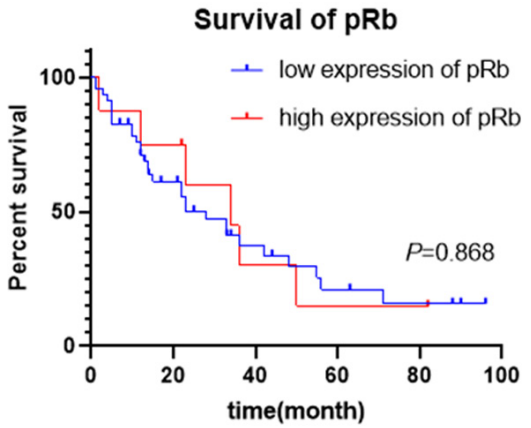

Figure 6. Kaplan-Meier survival curves of the different expression of pRb.

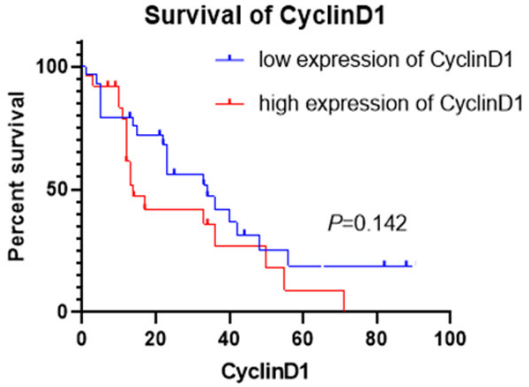

Figure 7. Kaplan-Meier survival curves of the different expression of CyclinD1.

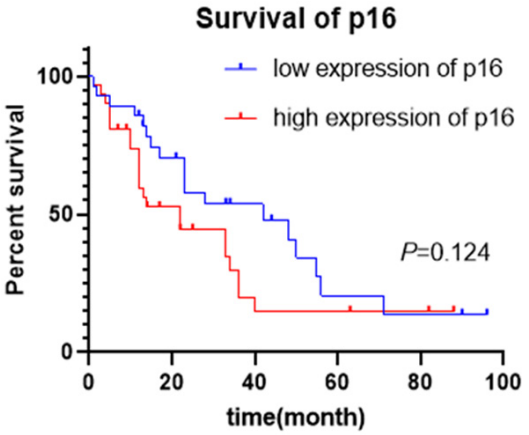

Figure 8. Kaplan-Meier survival curves of the different expression of p16.

through the E2F family of transcription factors that coordinate cell-cycle progression, nucleotide biosynthesis, DNA replication, mitotic progression, and DNA damage repair [8]. Further regulation occurs through a negative feedback loop whereby the CDK4 (or CDK6) inactivation of Rb relieves the Rb-mediated repression of the cyclin dependent kinase inhibitor 2A (CDKN2A gene; $p16^{INK4a}$ protein), which in turn leads to a reduction in CDK4/6 activity. This feedback loop effectively works as a natural brake on the activation of the $p16^{INK4a}$-CDK4-pRb pathway. It is also plausible that increasing the activity of CDK4 by mutation or increased expression of its binding partner CyclinD1 or CDK4 itself may overcome this negative feedback control and further activate the pathway. This process may generate dependency on CDK4 for the maintenance of cell proliferation.

$p16^{INK4a}$-CDK4-pRb is the downstream pathway of tumor suppressor genes and is closely related to the regulation of the cell cycle [9] and the regulation of cyclinD-dependent kinase 4 (CDK4) by CDK4-activating kinase. CDK4 is a factor that plays an important role in regulating the proliferation, differentiation, growth, and survival of melanocytes. Thus, changes in the expression of this protein or in the subset of transcription factors that it regulates may lead to different consequences such as cell growth or death.

The mutations and genomic alterations that are thought to cause melanoma are mainly involved in four groups of signaling pathways: the RAS-RAF-MAPK pathway, the PI3K-AKT (combined PTEN) pathway, or the $p16^{INK4a}$-CDK4-pRb and ARF-MDM2-p53 pathway [10]. The RAS-RAF-MAPK pathway is the most important pathway

for melanoma. The BRAF gene (the most common subtype of RAF) is most closely related to melanoma. The BRAF V600E mutation is the most classical, thereby enhancing the activity of the RAS-RAF-MAPK pathway in melanoma cells. In addition, this mutation site is an effective therapeutic target for cutaneous melanoma.

BRAF/MEK inhibitor therapy has been most effective in patients with BRAF V600 mutations [11, 12]. Unfortunately, the great majority of patients with melanoma who receive MAPK inhibition will progress, and more-effective, targeted therapies are needed. One possible strategy is dually targeting MAPK and the cyclin dependent kinases 4 and 6 (CDK4/6) [13]. Additionally, most Asian populations have non-CSD melanoma (acral and mucosal melanoma), and the MM BRAF mutation rate is less than 10% [14]. It has been suggested that the RAS-RAF-MAPK pathway may not play a leading role in MM. In our study, (19/54, 35.2%) of MM patients had high expression of CDK4, (35/54, 64.8%) MM patients had low expression of CDK4, and patients with CDK4 high expression had unfavorable three year survival rates (**Figure 5**) ($P < 0.05$). High expression of CDK4 were significantly associated with prognosis, suggesting that the $p16^{INK4a}$-CDK4-pRb pathway may play a more important role in MM, and the prognostic effect of cell cycle regulation on MM should not be ignored. CDK4/6 may represent a valid therapeutic target for cancer treatment in a broad spectrum of solid tumors including melanoma. Patients previously treated with MAPK and CDK4/6 inhibition may find this a more effective approach with mucosal melanoma.

The $p16^{INK4a}$-CDK4-pRb pathway also plays an important role in tumor suppression, and approximately 90% of melanomas have an imbalance in this pathway [15]. In the melanoma familial study, the CDK4 and $p16^{INK4a}$ genes are frequently found on the CDKN2A sites of germ cells carrying melanoma mutant genes, which may lead to weaker surveillance of melanoma cells [10]. Richard's study found that a gain of CDK4 and/or a loss of CDKN2A was associated with worse melanoma-specific survival [6]. This hypothesis is supported by the observation that 251 patients with primary cutaneous melanoma with CCND1 gain together with a loss of CDKN2A and/or a gain in CDK4 had a significantly poorer survival rate. Their data suggested that this amplified CDK4 pathway signaling is a common early event in melanoma and is more common in wild type tumors for BRAF and NRAS and may play a role in disease aggressiveness. Furthermore, it raises the possibility that in melanoma, CDK4 pathway activation may provide an additional oncogenic addiction irrespective of the BRAF or NRAS mutant status.

We found that patients with low expression of CDK4 were significantly older ($P < 0.05$) compared to patients with high expression of CDK. Moreover, patients with high expression of CDK4 were significantly associated with a worse prognosis. Our results agree with the viewpoint that melanoma in the elderly is known to present later, and therefore potentially has a worse prognosis than in younger patients [16]. The $p16^{INK4a}$-CDK4-pRb pathway may play a more important role in younger patients with MM.

Clearly, we need to complete the follow-up of this group of patients to achieve a final conclusion about the utility of these markers in this rare tumor. There will be other groups of patients with different cancers that can be fully staged, where this combination of markers may provide useful prognostic information.

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Disclosure of conflict of interest

None.

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