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Expression of c-Kit, p-ERK and cyclin D1 in malignant melanoma: An immunohistochemical study and analysis of prognostic value

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ABSTRACT

Background: The mitogen-activated protein kinase (MAPK) signaling pathway is one of the major cascades that are crucial for the initiation and progression of melanoma; however, the influence of these signaling molecules on patient survival has not been clarified.
Objective: The purpose of this study was to analyze the protein expression of MAPK signaling molecules in melanoma, and to correlate the expression status with clinicopathologic parameters.
Methods: Expression of c-Kit, phosphorylated ERK (p-ERK), and cyclin D1 was examined by immunohistochemistry in 78 primary melanomas, 24 metastatic lesions, and in 42 benign nevi. The following clinicopathologic variables were evaluated: age, gender, histologic type, tumor site, Breslow thickness, Clark's level, ulceration, and survival period. Statistical analyses were performed for assessment of associations and melanoma-specific survival.
Results: The expression of c-Kit, p-ERK, and cyclin D1 was significantly higher in primary melanomas than in nevi. c-Kit immunoreactivity was highest in thin (Tis-pT2) melanomas, and showed a significant reduction with tumor progression and metastasis. The expression of p-ERK was high in all stages of melanoma. Cyclin D1 positivity increased significantly according to tumor progression, but decreased in metastases. A significant correlation between p-ERK and cyclin D1 expression was observed. Survival analysis failed to detect any trends towards shorter or longer survival among patients expressing either c-Kit, p-ERK or cyclin D1.
Conclusions: The expression of c-Kit, p-ERK, and cyclin D1 might help to differentiate thin melanoma from melanocytic nevus, but it appears to lack prognostic potential.

1. Introduction

The mitogen-activated protein kinase (MAPK) pathways are evolutionally conserved signaling modules by which cells transduce extracellular signals into intracellular responses [1]. The prototypical MAPK pathway is the extracellular-signal-related kinase 1 (ERK1)/2 pathway, the central regulator of cell proliferation, which is reported to be involved in many types of cancer, including malignant melanoma (MM) [2,3]. Activating mutations and/or copy-number changes in signal transducing molecules in the MAPK pathway such as BRAF, NRAS, c-Kit, cyclin D1, and CDK4 have been identified in the distinct subtypes of MMs [4–7], indicating their potential as therapeutic targets.

c-Kit, a 145-kDa transmembrane receptor tyrosine kinase, plays a pivotal role in melanocyte migration, development and proliferation [8]. c-Kit activation is linked to cell proliferation via RAF/ERK cascade [9]. Although c-Kit has a clear effect on normal melanocyte proliferation, its role in melanoma tumorigenesis has been controversial [3]. Several studies have shown that c-Kit expression tended to be lost during melanocyte transformation, local melanoma growth, and metastasis [10–16]. Interestingly, however, mutations and/or copy-number increases in the *c-kit* gene have been identified in mucosal and acral melanomas and in melanomas occurring on skin with chronic sun-induced damage, indicating that *c-kit* is an important oncogene in such melanoma subtypes [4,6,17,18].

The ERK pathway is activated by phosphorylation cascades and participates in signal transduction pathways that control embryogenesis, cell differentiation, cell proliferation, and cell death [19]. Numerous stimuli, including hormones, growth factors, and differentiation factors, activate the ERK pathway [19]. Upon activation, ERK1/2 either regulates cytoplasmic targets or translocates to the nucleus, where they induce the phosphorylation of several transcription factors and drive cell proliferation through the

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regulation of cyclin D1 expression [20]. Cyclin D1, a 45-kDa protein encoded by the CCND1 gene (11q13) and a member of the G1 cyclin family involved in regulating the G1–S transition of the cell cycle [21], is one of the important downstream effectors of the ERK1/2 pathway. It mediates phosphorylation and functional inactivation of retinoblastoma protein in association with the cyclin-dependent kinases (CDK) 4 and CDK6, leading to the release of various transcription factors such as E2F family members, and promotes entry into mitosis [22,23]. Although the role of cyclin D1 was less established in MM, recent studies have demonstrated the frequent amplification (up to 45%) of the CCND1 gene in acral melanomas [7,24,25]. In addition, antisense-mediated knockdown of CCDN1 caused apoptosis in vitro and led to significant tumor shrinkage of melanoma xenografts in mice, thus confirming the role of CCDN1 as a melanoma oncogene [25].

Although the importance of the MAPK signaling factors including c-Kit, p-ERK, and cyclin D1 in MM is well recognized, there are only a few reports regarding their association with other variables and their influence on the clinical outcome in Japanese patients, for whom more than half were revealed as acral lentiginous melanoma (ALM). The present study was designed to compare the immunohistochemical expression of c-Kit, p-ERK, and cyclin D1 between in MM and in nevus, and to evaluate its prognostic potential in patients with MM in Japan.

2. Materials and methods

2.1. Patients

In all, 78 patients with MM observed at the Department of Dermatology, Kyushu University (Fukuoka, Japan) between the years 1999 and 2009 were included in this study, which was approved by the Institutional Ethics Committee. Clinical and demographic information was retrieved from each patient's file, and all cases were reviewed by two dermatopathologists who were blind to information on the lesion site, age, and gender of the patients. Ulceration was scored as present or absent. We applied published guidelines for reporting immunohistochemistry-based tumor marker studies [26].

2.2. Immunohistochemistry

Immunohistochemical analyses were performed using formalin-fixed and paraffin-embedded archival tissues. We performed immunostaining on 3–4-μm-thick tissue sections by the Universal Immuno-enzyme Polymer method (Histofine® Simple Stain AP, Nichirei, Tokyo, Japan). The sections were deparaffinized with xylene for 20 min and rehydrated through graded ethanol solutions. Antibody-binding epitopes were retrieved using an autoclave unmasking process (10 min at 110 °C in 10 mmol/L citrate buffer, pH 7.0), and nonspecific binding was blocked using 10% goat serum. Then, the sections were incubated with rabbit polyclonal antibody against c-Kit (CD117) (A4502, Dako, Tokyo, Japan) in Tris-buffered saline (TBS) for 30 min at room temperature at a dilution of 1:500, or with rabbit monoclonal antibody against p-ERK1/2 (20G11, Cell Signaling Technology, Beverly, MA, USA) or mouse monoclonal antibody against cyclin D1 (DSC6, Cell Signaling Technology) at 4 °C overnight in TBS at a dilution of 1:100. Immunodetection was conducted with an alkaline phosphatase detection system according to the manufacturer's instructions, followed by counterstaining with hematoxylin. For c-Kit staining, mast cells were used as internal positive controls. For p-ERK and/or cyclin D1 immunohistochemistry, sections of extramammary Paget disease known to express p-ERK and/or cyclin D1 served as external positive controls. Replacement of the various primary antibodies by unrelated IgG rabbit or mouse antibodies served as negative controls. All incubations were carried out at room temperature. Washes with TBS were performed between each step according to the manufacturer's protocols. Semiquantitative assessment of expression was performed according to the following scale: −, <5% of the tumor cells were positive; +, 5–25% of the tumor cells were positive; 2+, 26–50% of the tumor cells were positive; and 3+, >50% of the tumor cells were positive. The slides were scored for staining positivity and stain intensity by two independent observers without prior knowledge of the clinical outcome.

2.3. Statistical analysis

All statistical analyses were performed using the SPSS statistical software package for Windows (Version 11.0, SPSS Inc., Chicago, IL, USA). Risk factors (covariates) were considered dichotomous for c-Kit, p-ERK, and cyclin D1 expression (negative vs. positive), gender (male vs. female), and tumor subtype (ALM vs. non-ALM); age and Breslow thickness were calculated as continuous variables; and Clark's level was considered as an ordinal variable. The 95% confidence interval for survival was calculated and reported for the multivariate statistical model. A p-value less than 0.05 was regarded as statistically significant.

3. Results

3.1. Patient data

Comprehensive clinical and histopathological data is given in Table 1. The patients, 32 men and 46 women, ranged in age from 32

Table 1
Patients' characteristics.

Parameters	Number (%)
Gender	
Male	32 (41.0)
Female	46 (59.0)
Age in years	
Mean ± SD	62.6 ± 15.2 (range, 32–89)
Median	64.5
Breslow thickness	
Tis	12 (15.4)
T1	16 (20.5)
T2	9 (11.5)
T3	21 (27.0)
T4	20 (25.6)
Mean ± SD (mm)	3.2 ± 3.8 (range, 0.1–24.5)
Median (mm)	2.25
Clark's level	
I	12 (15.4)
II	12 (15.4)
III	15 (19.2)
IV	20 (25.6)
V	19 (24.4)
Tumor subtype	
ALM	49 (62.8)
SSM	15 (19.2)
LMM	5 (6.4)
NM	7 (9.0)
Mucosal	2 (2.6)
Tumor site	
Extremity	58 (74.4)
Trunk	8 (10.3)
Head and neck	9 (11.5)
Genital area	3 (3.8)
Ulceration	
Present	28 (35.9)
Absent	50 (64.1)
Survival time in months	
Mean ± SD	45.9 ± 27.0 (range, 4–110)
Median	40

ALM: acral lentiginous melanoma; SSM: superficial spreading melanoma; NM: nodular melanoma; LMM: lentigo maligna melanoma; Mucosal: mucosal melanoma.