High expression of cytoplasmic phosphorylated CSE1L in malignant melanoma but not in benign nevi: phosphorylated CSE1L for the discrimination between melanoma and benign nevi

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Abstract: Melanoma is difficult to treat when it has metastasized. Discrimination between melanoma and benign nevi in melanocytic lesions is crucial for identifying melanomas and consequently improving melanoma treatment and prognosis. The chromosome segregation 1-like (CSE1L) protein has been implicated in cancer progression and is regulated by phosphorylation by extracellular signal-regulated kinase 1/2 (ERK1/2) signaling, a critical pathway in melanoma progression. We studied phosphorylated CSE1L expression in human melanoma and benign nevi specimens. Immunohistochemistry with tissue microarray using antibody against phosphorylated CSE1L showed that melanomas exhibited considerable staining for phosphorylated CSE1L (100%, 34/34), whereas the benign nevi showed only faint staining (0%, 0/34). Melanomas mainly exhibited cytoplasmic phospho-CSE1L distribution, whereas the benign nevi mainly exhibited nuclear phospho-CSE1L distribution. Moreover, immunohistochemistry with anti-CSE1L antibody revealed that CSE1L mainly exhibited cytoplasmic/nuclear distribution and nuclear distribution was the dominant. Immunofluorescence with B16F10 melanoma cells showed cytoplasmic distribution of phospho-CSE1L and nuclear distribution of CSE1L. Our results indicated that nuclear CSE1L is mainly non-phosphorylated CSE1L and is involved in gene regulation and cytoplasmic CSE1L is mainly phosphorylated CSE1L and is involved in cytoplasmic signaling regulation in melanocytic tumorigenesis. Furthermore, immunohistochemical analysis of cytoplasmic phospho-CSE1L may aid in the diagnosis of melanoma.

Keywords: CSE1L, cytoplasmic, melanoma, nuclear, phospho-CSE1L, phosphorylation

Introduction

Melanomas cause most skin-cancer-related deaths. These malignancies can develop in normal skin or from atypical nevi. Nevi are benign tumors derived from melanocytes, and the risk of the transformation of a common nevus into a melanoma is low. However, the atypical nevi are potential precursors for melanoma. Melanomas are highly invasive, but early-stage melanomas are highly curable; however, advanced-stage and metastatic melanomas are more difficult to treat and are often fatal. Thus, the distinction between melanomas and benign nevi is necessary for identifying melanocytic lesions and consequently melanoma treatment and prognosis for a more satisfactory management [1]. Immunohistochemistry is usually of limited value in the diagnosis and discrimination of melanoma and certain types of nevi, such as atypical Spitz nevi, recurrent melanocytic nevi, and proliferative nodules in congenital nevi [2]. The chromosome segregation 1-like (CSE1L) protein is the human homolog of the yeast chromosome segregation protein CSE1 [3]. Pathological studies have shown
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that CSE1L is highly expressed in cancer and is associated with advanced stage and poor patient prognosis [4]. A pathological study reported that CSE1L expression predominantly correlated with advanced stages of melanoma [5]. Our recent studies showed that CSE1L is a phosphorylated protein and CSE1L phosphorylation is regulated by Ras-ERK signaling [6, 7]. The extracellular signal-regulated kinase 1/2 (ERK1/2) is a major downstream transducer of Ras and plays a crucial role in the progression of various cancers including melanoma [8-11]. Thus, phosphorylated CSE1L may be involved in the development of melanomas. In this study, we report that CSE1L mainly exhibited cytoplasmic/nuclear distribution and nuclear distribution was the dominant; phosphorylated CSE1L mainly exhibited cytoplasmic distribution, whereas benign nevi exhibited nuclear phospho-CSE1L distribution. Moreover, phosphorylated CSE1L was significantly stained in melanomas but showed faint staining in benign nevi. Immunohistochemical analysis of phospho-CSE1L expression may be helpful in discriminating melanomas from benign nevi.

Materials and methods

Production of antibodies specific to phosphorylated CSE1L

Non-phosphopeptide LTEYLKKTLDPPAC and phosphopeptide LTpYEpLKKTLDPDPAC (where Tp denotes phosphothreonine and Yp denotes phosphotyrosine) were synthesized using the solid-phase method. The phosphorylated peptides were conjugated through the N-terminal cysteine thiol to keyhole-limpet hemocyanin (KLH). New Zealand rabbits were immunized five times with the phosphopeptides. The immune serum was collected a week after the final immunization. The IgG fractions were purified using a protein G column (Amersham Pharmacia Biotech, Uppsala, Sweden). The antibodies were purified using the phosphorylated peptide affinity column and then through non-phosphopeptide cross-adsorption to remove nonphospho-specific antibodies. The titer and the specificity of the antibodies were tested using enzyme-linked immunosorbent assay (ELISA) and immunoblotting.

Cells and cell treatments

The procedure for producing B16-Ras cells (B16F10 mouse melanoma cells transfected with a v-H-ras-expressing vector) and B16-dEV cells (B16F10 mouse melanoma cells transfected with control vectors) was as previously described [6]. The cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum, 100 unit/mL of penicillin, 100 mg/mL of streptomycin, and 2 mmol/L glutamate at 37°C in a humidified 5% CO2 atmosphere. The cells were maintained in media containing 200 μg/mL of G418. For the experiments, the cells were cultured in media without G418.

Immunoblotting

The cells were washed with phosphate-buffered saline (PBS) and lysed in an ice-cold radioimmunoprecipitation assay buffer (25 mM Tris-HCl (pH 7.2), 0.1% SDS, 0.1% Triton X-100, 1% sodium deoxycholate, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 5 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL of aprotinin, and 5 μg/mL of leupeptin) containing phosphatase inhibitors (25 mM β-glycerophosphate and 5 mM sodium fluoride). Protein concentrations were determined using a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). Proteins were transferred to nitrocellulose membranes (Amersham Pharmacia, Little Chalfont, Buckinghamshire, UK), and immunoblotting was performed using an anti-CSE1L antibody (clone...
Phospho-CSE1L expression in melanoma

Table 1. Clinical-pathological correlation of phosphorylated CSE1L expression in melanomas

<table>
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<tr>
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<tr>
<td></td>
<td>Weak</td>
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<td>Total</td>
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<td>M</td>
<td>4 (23.5)</td>
<td>13 (76.5)</td>
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<td>6 (42.9)</td>
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<td>15 (75)</td>
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<td>3 (75)</td>
<td>1 (25)</td>
<td>4</td>
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<td>V</td>
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<td>6 (50)</td>
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Abbreviations: F, female; M, male. P value was calculated using a Pearson chi-square test.

Patients

The study was approved by the Ethics Committees of Changhua Christian Hospital (Changhua, Taiwan) and it adhered to the guidelines approved by the institutional review board of the hospital. Melanoma is relatively rare in Asian populations compared with fair-skinned populations. Melanoma specimens were obtained from 34 consecutive patients who had recently been diagnosed at our hospital. The tumors were graded and categorized according to the Staging Manual of the American Joint Committee on Cancer (7th edition) [12]. The study enrolled 20 benign-nevi cases. The patient group comprised 17 men and 17 women, with a mean age of 75.4 years (range: 47-100 years). Twenty patients had ulcers in their tumors. Four, 23, and 7 cases were classified as Clark’s Level III, IV, and V tumors, respectively.

Tissue microarrays and immunohistochemistry

Tissue microarrays and immunohistochemistry were performed as previously described [13]. For the tissue microarrays, three cores from cancerous tissues and one core from benign nevi in each paraffin block were longitudinally cut and arranged into new paraffin blocks. Immunohistochemistry was performed on 6-μm tissue sections fixed in formalin and embedded in paraffin by using a 100-fold dilution of anti-phospho-CSE1L antibodies or anti-CSE1L antibodies (clone 3D8, Abnova, Taipei, Taiwan). Immunohistochemical detection was performed using a labeled streptavidin-biotin method with a Histostain kit according to the manufacturer’s instructions (Zymed, San Francisco, CA, USA). The sections were developed using diaminobenzidine, washed with distilled water, and counterstained with Mayer’s hematoxylin.
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Statistical analyses

Results of clinicopathological variables were tested using Fisher’s exact test to ascertain whether differences between the groups were statistically significant. Analyses were performed using the Statistical Package for Social Sciences (SPSS, Version 15.0; SPSS Inc., Chicago, IL, USA). A $P$ value of $< 0.05$ (two-tailed test) was considered statistically significant.

Results

Antibodies specific to phosphorylated CSE1L were produced by immunizing New Zealand rabbits with synthetic phosphopeptides designed to correspond to the putative phosphorylation domain of CSE1L. The results of immunoblotting with cell lysates from B16-Ras cells showed that the anti-phospho-CSE1L antibodies recognized phosphorylated CSE1L (Figure 1). We then studied phosphorylated CSE1L expression in human melanoma and benign-nevi specimens. We observed that no significant clinical-pathological correlation of phospho-CSE1L expression in melanomas (Table 1). Compared with fair-skinned populations, the occurrence of melanoma is relatively rare in Asian populations. This apparent correlation (no significant clinical-pathological correlation of phospho-CSE1L expression in melanomas) may have been due to the low number of cases in this study. However, we observed a nonsignificant trend, reflecting the relation of phospho-CSE1L expression with ulceration and lymph node metastasis of melanoma (Table 1). Ulceration of a cutaneous melanoma on microscopic sections is an adverse prognostic finding [14]. An analysis of the ulcerated tumors showed that 10 of 15 cases with high phospho-CSE1L expression showed ulcerated tumors (Table 1). In addition, six of seven cases with high phospho-CSE1L expression showed lymph node metastasis (Table 1).

Figure 2. Representative immunohistochemical images of phospho-CSE1L expression in human nevi. A, B. Hematoxylin and eosin staining. C, D. Phospho-CSE1L staining with antibody against phosphorylated CSE1L. Original magnification: A and C. ×100; B and D. ×400.
Immunohistochemistry results showed that phospho-CSE1L was faintly stained in benign nevi (0/20) (Figure 2). Immunohistochemical staining indicated that all melanomas (100%, 34/34) exhibited significant positive phospho-CSE1L staining (Figure 3). The finding that cytoplasmic phospho-CSE1L was highly expressed in human melanomas but was faintly stained in benign nevi indicated that phospho-CSE1L plays a role in the development of melanoma. Moreover, most of the melanomas mainly exhibited cytoplasmic phospho-CSE1L staining, whereas most benign nevi exhibited nuclear phospho-CSE1L staining. The result of immunohistochemical staining with antibody against CSE1L (clone 3D8) revealed that cellular total CSE1L showed both cytoplasmic and nuclear distribution and nuclear distribution was the dominant (Figure 4). In addition, immunofluorescence analysis of the distribution of phospho-CSE1L expression in B16F10 mouse melanoma cells with antibody against phosphorylated CSE1L showed that phospho-CSE1L was mainly distributed in the cytoplasm of B16-Ras melanoma cells, whereas CSE1L was mainly distributed in the nucleus as analyzed using anti-CSE1L antibodies (clone H2) (Figure 5). The results indicated that the cytoplasmic distribution of phospho-CSE1L plays a role in the development of melanoma. In addition, analysis of nuclear and cytoplasmic distribution of phospho-CSE1L may be useful in distinguishing melanomas from benign nevi.

**Discussion**

The Ras-Raf-MEK-ERK signaling plays a crucial role in melanoma progression [10, 11]. Ras-Raf-MEK-ERK pathway was activated in over 80% of cutaneous melanomas [15]. Studies have reported that phospho-ERK expression is significantly higher in primary melanomas than in nevi and that phospho-ERK expression is high in all stages of melanoma [10, 11, 16].
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CSE1L is highly expressed in most cancers and in vitro cell-line studies have reported that CSE1L phosphorylation is regulated by Ras-Raf-ERK signaling [6, 7]. The present study showed that melanomas exhibited significant phospho-CSE1L staining, whereas benign nevi exhibited faint staining of phospho-CSE1L (Figures 2 and 3). Because ERK signaling is highly activated in melanomas, our results are consistent with those of in vitro cell-line studies in that ERK signaling and phosphorylated CSE1L might be involved in melanocytic tumorigenesis.

CSE1L has been reported to associate with chromatin and regulates expression of select p53 target genes, thus, nuclear CSE1L can bind select genes with significant functional consequences for p53-mediated transcription and determine cellular outcome [17]. It was also reported that nuclear accumulation of CSE1L may implicate in the nuclear concentration of transcription factors conveying prooncogenic signals [18]. High CSE1L nuclear staining was reported to be associated with a lower overall survival rate in bladder urothelial carcinomas [19]. On the other hand, cytoplasmic CSE1L was reported to be correlated with the depth of tumor penetration and cancer stage of colorectal cancer [20]. Cytoplasmic CSE1L was also reported to regulate protrusion extension, microvesicle formation, and was implicated in the migration and invasion of cancer cells [6, 7, 21]. Our present results showed that melanomas mainly exhibited cytoplasmic phospho-CSE1L distribution and CSE1L mainly exhibited cytoplasmic/nuclear distribution and nuclear distribution was the dominant (Figures 3 and 4). These results indicated that nuclear CSE1L was mainly non-phosphorylated CSE1L and was involved in gene regulation and cytoplasmic phospho-CSE1L was mainly phosphorylated CSE1L and was involved in cellular tumor progression signaling in melanocytic tumorigenesis.

Figure 4. Representative immunohistochemical images of CSE1L expression in melanomas. A, B. Hematoxylin and eosin staining. C, D. CSE1L staining with antibody against CSE1L (clone 3D8). Original magnification: A and C. ×100; B and D. ×400.
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Standard histopathology is a common method for diagnosing melanocytic tumors; nevertheless, equivocal diagnosis may exist in a subset of cases based on histomorphological features alone [2, 22-25]. Representative examples of those cases include atypical Spitz nevi; recurrent melanocytic nevi; melanocytic nevi of the genital, acral, or mammary regions; proliferative nodules in congenital nevi; and recently sun-exposed nevi, in which diagnostic ambiguity of a melanoma and a nevus may arise [26-33]. Misdiagnosing a melanoma as a benign nevus results in insufficient treatment. Conversely, misdiagnosing a benign nevus as a melanoma results in unnecessary skin destruction caused by overly aggressive treatment [34]. Our results showed that most of the melanomas exhibited cytoplasmic phospho-CSE1L distribution, whereas the benign nevi mainly exhibited nuclear phospho-CSE1L distribution (Figures 2 and 3). Our results suggested that cytoplasmic phosphorylated CSE1L is associated with melanocytic tumorigenesis and immunohistochemical analysis of cytoplasmic

Figure 5. Representative micrographs showing phospho-CSE1L distribution in the cytoplasm of B16-Ras cells. Immunofluorescence shows cytoplasmic distribution of phospho-CSE1L analyzed with anti-phospho-CSE1L antibodies and nuclear distribution of CSE1L analyzed with anti-CSE1L antibodies (H2). Bar = 20 μm.
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Phospho-CSE1L may be helpful in diagnosing microscopically ambiguous cases and facilitate patient-management decisions.

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Disclosure of conflict of interest

None.

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