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Original Article

Oncogenic Ras expression increases cytoplasmic distribution and phosphorylation of CSE1L in B16F10 melanoma cells

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Abstract: Ras activation confers the transformation activities of melanocytic lesions and malignant progression of melanoma. The *CSE1L/CAS* (chromosome segregation 1-like/cellular apoptosis susceptibility) gene is located in 20q13, a chromosomal region that correlates with melanoma development. CSE1L is highly expressed in melanoma and correlated with cancer stage and the poor prognosis of the disease. We studied the relation of Ras activation and the cytoplasmic distribution and phosphorylation of CAS in melanoma cells. Immunoblotting showed B16F10 melanoma cells overexpressing Ras increased CSE1L phosphorylation. Immunofluorescence showed that phosphorylated CSE1L mainly distributed in the cytoplasm of B16F10 melanoma cells, and Ras overexpression resulted in increased cytoplasmic distribution of phosphorylated CSE1L. CSE1L knockdown decreased the phosphorylation of the extracellular signal-regulated kinase1/2 (ERK1/2) induced by Ras in B16F10 melanoma cells. In immunohistochemistry, the tumor cells of melanoma showed strong cytoplasmic phosphor-CSE1L staining, while nevus cells showed weak cytoplasmic phosphor-CSE1L staining. Our results indicated that phosphorylated CSE1L plays a role in modulating the signaling pathway and progression of melanoma harboring *Ras* mutation.

Keywords: CAS, CSE1L, cytoplasmic, nuclear, melanoma, phosphorylation, Ras

Introduction

Melanoma is the most serious type of skin cancer which forms from melanocytes. Malignant melanoma is highly dangerous. Melanomas with *Ras* mutation are especially difficult to treatment due to its metastatic activity and the development of drug resistant ability [1]. Oncogenic *Ras* activation triggers cytoplasmic tumor progression signaling, this can directly stimulates the malignant progression of melanocytic lesions, or this may in turn transduce signal into the nuclei to activate melanoma progression related genes [2, 3]. Thus, studying the change in the status of cellular signaling or tumor progression-related proteins induced by *Ras* in melanoma should be helpful for the development of reagent (drug) for melanoma treatment. The extracellular signal-regulated kinase1/2 (ERK1/2) is a major downstream transducer of Ras [4]. ERK1/2 activation is suspected to play a critical role in the malignant progression of melanomas induced by Ras [5-7]. ERK1/2 activation activates various signaling and genes that contribute to melanoma progression [8].

The CSE1L/CAS (chromosome segregation 1-like/cellular apoptosis susceptibility) protein is the human homologue of CSE1, the yeast chromosome segregation protein [9]. *CSE1L* is located on the 20q13 chromosomal region, a region that frequently harbors amplifications that correlate with the development of various

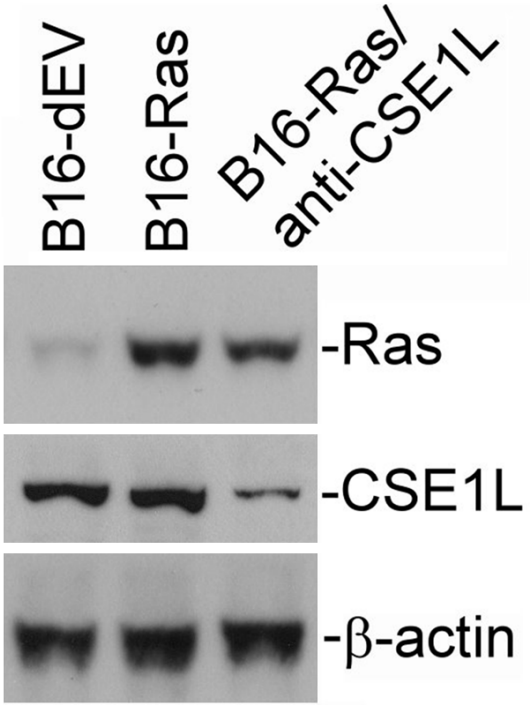

Figure 1. The levels of Ras and CSE1L expression in B16-dEV, B16-Ras, and B16-Ras/anti-CSE1L cells analyzed by Western blotting analysis with anti-CSE1L antibodies and anti-Ras antibodies. β-actin levels were assayed as a control.

cancers including melanoma [10-12]. Studies have also showed that the amplification of chromosome 20q is implicated in the tumorigenic transformation of melanocytes [13-15]. Pathological studies have showed CAS is highly expressed in most cancers including melanoma, and its expression is correlated with the grade and stage of melanoma [16, 17]. CSE1L is a phosphorylated protein and the phosphorylation of CSE1L is regulated by the Ras/ERK signaling pathway [18-23]. In this study, we analyzed the relation of Ras activation and cytoplasmic distribution and phosphorylation of CSE1L in melanoma cells. Our results showed that Ras regulated the cytoplasmic/nuclear distribution of phosphorylated CSE1L. The results indicated that the cytoplasmic phosphorylated CSE1L is implicated in tumor progression in melanoma harboring *Ras* mutation.

Materials and methods

Antibodies

The antibodies used in the experiment were anti-p21/ras (EP1125Y) and anti-α-tubulin (EP1332Y) (Epitomics, Burlingame, CA, USA); anti-CSE1L (3D8) and anti-phospho-ERK1/2 (Abnova, Taipei, Taiwan); anti-β-actin (Ab-5) (Lab Vision, Fremont, CA, USA); anti-CSE1L (clone H2) and anti-ERK1/2 (MK1) (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Production of antibodies specific to phospho-CSE1L

Phosphopeptide, LT^{p}EY^{p}LKKTLDPDPAC (T^{p} denotes phosphothreonine and Y^{p} denotes phosphotyrosine), and nonphosphopeptide, LTEY-LKKTLDPDPAC, were synthesized using the solid phase method. The phosphorylated peptides were conjugated through the N-terminal cysteine thiol to keyhole limpet haemocyanin (KLH). New Zealand rabbit was immunized five times with the peptides. The immune serum was collected one week after the last immunization. The IgG fractions were purified using a protein G column (Amersham Pharmacia Biotech, Uppsala, Sweden). The antibodies were purified by a phosphorylated peptide affinity column and then with non-phosphopeptide cross-adsorption to remove non-phospho-specific antibodies. The titer and the specificity of the antibodies were tested by ELISA and immunoblotting.

Cells and DNA transfections

B16F10 melanoma cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/mL of penicillin, 100 mg/mL of streptomycin, and 2 mmol/L of glutamate at 37°C under a humidified 5% CO_2 atmosphere. Cells were separately transfected with the control pZIP-NeoSV(X) empty vector plus control shRNA plasmids (sc-108060; Santa Cruz Biotechnology, Santa Cruz, CA, USA), pZIP-v-H-ras plasmids plus control shRNA plasmids, and pZIP-v-H-ras plus CSE1L shRNA plasmids (sc-29909-SH, Santa Cruz) to obtain B16-dEV, B16-Ras cells, and B16-Ras/anti-CSE1L cells respectively, by using the Lipofectamine plus reagent (Invitrogen, Carlsbad, CA, USA). Transfected cells were selected with 1 mg/ml G418 for 3 weeks. Multiple drug-resistant colonies (> 50) were pooled together and amplified in mass culture. Transfected cells were selected with 1 mg/ml G418 for 3 weeks then with 1 μg/ml

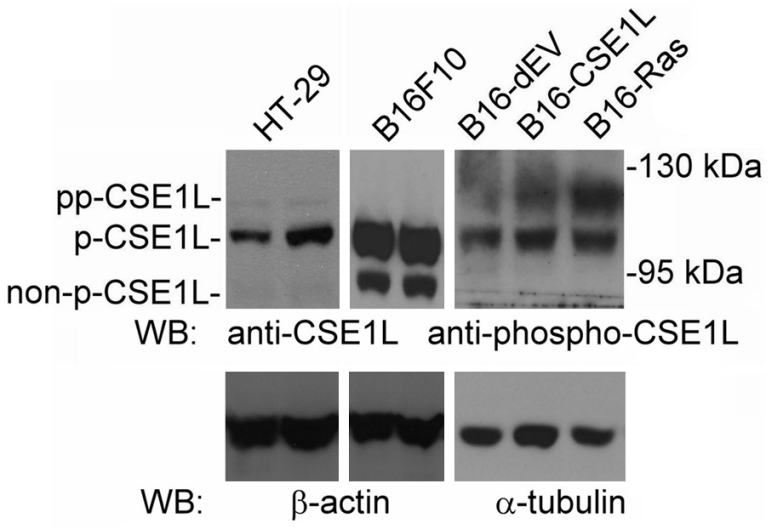

Figure 2. Ras activation increases hyper-phosphorylated CSE1L expression in B16F10 melanoma cells as analyzed by antibodies against CSE1L and phosphorylated CSE1L. The levels of phosphorylated and non-phosphorylated CSE1L in B16-dEV, and B16-Ras cell lysates prepared using lysis buffer containing protein phosphatase inhibitors and analyzed with anti-CSE1L antibody (clone 3D8) anti-phospho-CSE1L antibodies. β-actin levels were assayed as a control. pp-CSE1L: hyper-phosphorylated CSE1L; p-CSE1L: hypo-phosphorylated CSE1L; non-p-CSE1L: non-phosphorylated CSE1L. Note that anti-CSE1L antibody mainly recognized hypo-phosphorylated CSE1L and non-phosphorylated CSE1L; Ras activation increases hyperphosphorylated CSE1L expression.

puromycin for 3 weeks. For the experiments, cells were cultured in medium without G418.

Immunoblotting

Cells were washed with phosphate-buffered saline (PBS) and lysed in ice-cold radioimmunoprecipitation assay (RIPA) buffer [25 mM Tris-HC1 (pH 7.2), 0.1% SDS, 0.1% Triton X-100, 1% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 5 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 5 μg/ml leupeptin] containing phosphatase inhibitors (25 mM β-glycerophosphate and 5 mM sodium fluoride). The protein concentrations were determined with a BCA protein assay kit (Pierce, Rockford, IL, USA). Fifty micrograms of each protein sample was loaded onto SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose membranes (Amersham Pharmacia, Little Chalfont, Buckinghamshire, UK). The membrane was incubated blocking buffer [1% bovine serum albumin (BSA), 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, and 0.1% Tween-20] for 1 h. The blots were reacted at 4°C for overnight with primary antibodies followed by incubation with secondary antibodies conjugated to horseradish peroxidase for 1 h. The levels of protein were detected by enhanced chemiluminescence with a Forte Western HRP Substrate (Millipore, Billerica, MA, USA).

Immunofluorescence

Cells grown on coverslips (12 × 12 mm) were cytospun at 1000 rpm for 10 min. Cells were washed with PBS, fixed with 4% paraformaldehyde, permeabilized with methanol, and blocked with PBS containing 0.1% BSA. Samples were incubated with primary antibodies for 1 h. Samples were then washed three times with PBS and followed by incubating with goat anti-mouse (or anti-rabbit) IgG secondary antibodies coupled to Alexa Fluor 488. Cells were examined with a Zeiss Axiovert 200 M inverted fluorescence microscope (Carl Zeiss, Jena, Germany). Experiments were carried out on duplicate coverslips and ten random fields were imaged per coverslip.

Immunohistochemistry

Immunohistochemistry was performed on 6-μm formalin-fixed/paraffin-embedded cancer tissue sections using a 100-fold dilution of the anti-phospho-CSE1L antibodies. Immunohistochemical detection was performed with use of a labeled streptavidin-biotin method with the Histostain kit according to the manufacturer's instructions (Zymed, San Francisco, CA). Sections were developed with diaminobenzidine, washed with distilled water, and counterstained with Mayer's hematoxylin.

Results

To produce antibody specific to phosphorylated CSE1L, New Zealand rabbits were immuned with synthetic phosphopeptides designed to correspond to the putative phosphorylation domain of CSE1L. Antibodies from the immune

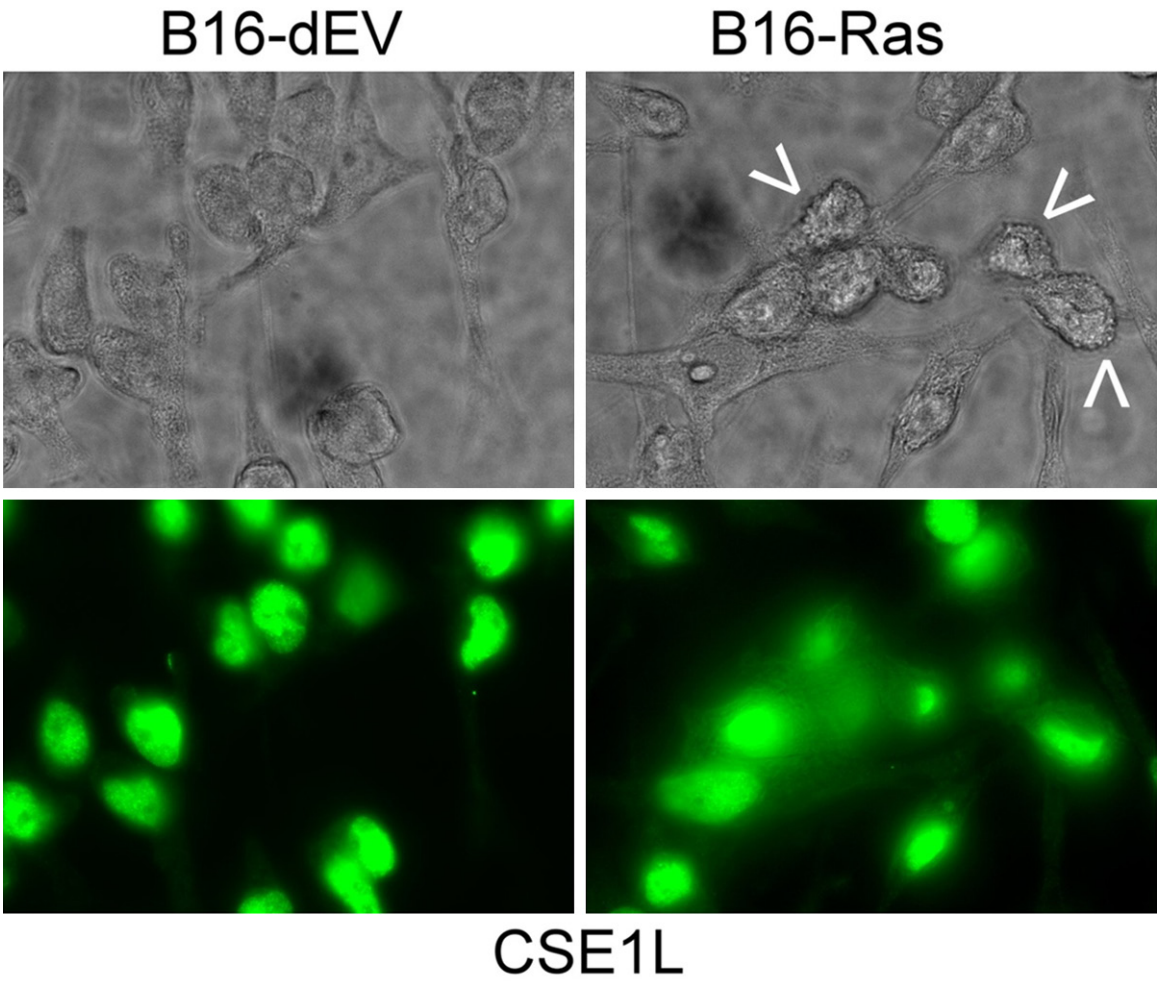

Figure 3. Ras activation increases the cytoplasmic distribution of CSE1L. Representative images showing v-H-Ras transfection increased the cytoplasmic distribution of CSE1L in B16F10 melanoma cells as analyzed by immunofluorescence with anti-CSE1L antibodies.

serum were purified with phosphorylated peptide affinity column and then with non-phosphopeptide cross-adsorption to remove non-phospho-specific antibodies. The phosphorylation of CSE1L is regulated by Ras/ERK [18-23]. The B16F10 melanoma cell line is commonly used in studying the tumorigenicity of melanoma [24, 25]. We established B16F10 melanoma cells overexpressing Ras to study the relation of Ras activation and the cytoplasmic distribution and phosphorylation of CSE1L in melanoma cells (**Figure 1**). Immunoblotting showed that anti-CSE1L antibodies (clone 3D8) recognized both phosphorylated CSE1L and non-phosphorylated CSE1L (**Figure 2**). The results of immunoblotting with cell lysates from B16-Ras melanoma cells showed that the anti-phospho-CSE1L antibody recognized phosphorylated CSE1L (**Figure 2**). These results indicated that the anti-phospho-CSE1L antibody can be used for studying the phosphorylation of CSE1L and its cytoplasmic distribution induced by Ras activation in melanoma cells.

The effect of Ras activation on the cytoplasmic distribution of phosphorylated CSE1L in melanoma cells was studied by immunofluorescence with B16-Ras cells and the control B16-dEV cells. The results of immunofluorescence with anti-CSE1L antibodies (clone H2) showed that B16-dEV cells mainly showed nuclear CSE1L distribution (**Figure 3**). Ras mutation activation by *v-H-Ras* overexpression in B16F10 melanoma cells resulted in increased cytoplasmic distribution of CSE1L (**Figure 3**). The results of immunofluorescence with anti-phospho-

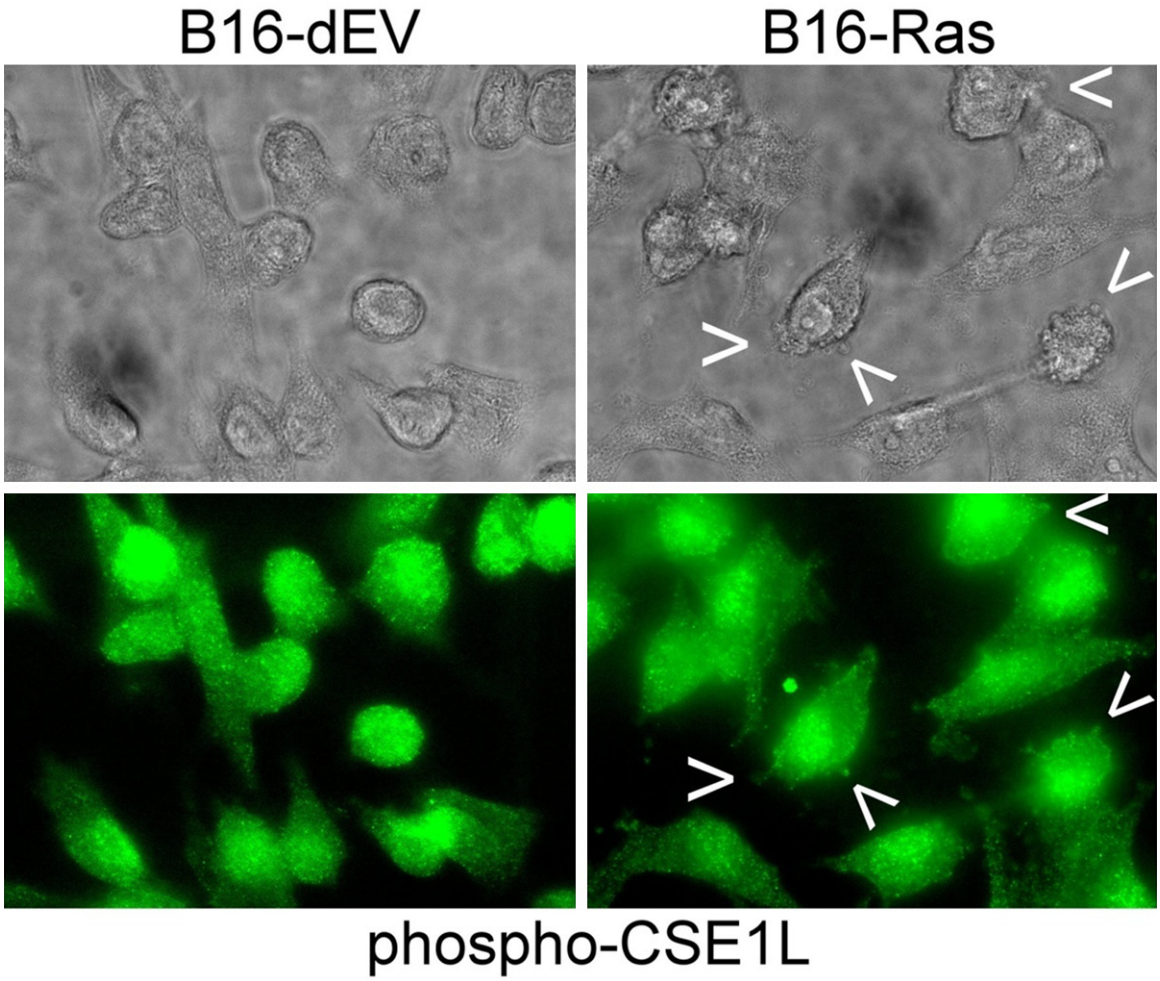

Figure 4. Ras activation increases the cytoplasmic distribution of phosphorylated-CSE1L. Representative images show v-H-Ras transfection increased the cytoplasmic distribution of phosphorylated-CSE1L in B16F10 melanoma cells as analyzed by immunofluorescence with antibodies against phosphorylated-CSE1L.

CSE1L antibodies showed that phosphorylated CSE1L was expressed in both the nuclear and cytoplasm of B16-dEV melanoma cells (**Figure 4**). *v-H-Ras* overexpression in B16F10 melanoma cells (i.e. B16-Ras cells) resulted in increased cytoplasmic distribution of phosphorylated CSE1L (**Figure 4**). Furthermore, Ras overexpression resulted in generation of microvesicles on the membranes of B16-Ras cells, and immunofluorescence with anti-CSE1L antibodies and anti-phospho-CSE1L antibodies showed the accumulation of phospho-CSE1L in the microvesicles on the membranes of B16-Ras cells (**Figure 4**).

The increased cytoplasmic distribution of phosphorylated CSE1L induced by Ras suggested that the phosphorylation and cytoplasmic distribution of CSE1L is regulated by Ras in tumor cells. ERK1/2 is an important tumor signaling and it lies downstream of Ras and plays a crucial role melanoma progression [8]. We have previously reported that the phosphorylation of CSE1L is regulated by the Ras/ERK signaling pathway in that Ras induced ERK-dependent phosphorylation of CSE1L [18, 19]. The regulation of Ras/ERK signaling by CSE1L was also conducted in this study. The results of immunoblotting with cell lysates from the B16-Ras/anti-CSE1L melanoma cells (i.e. B16-Ras cells transfected with CSE1L shRNA plasmids) showed that CSE1L knockdown inhibited phospho-ERK1/2 expression induced by Ras in B16F10 melanoma cells (**Figure 5**). The results indicated that CAS regulated the phosphorylation of ERK.

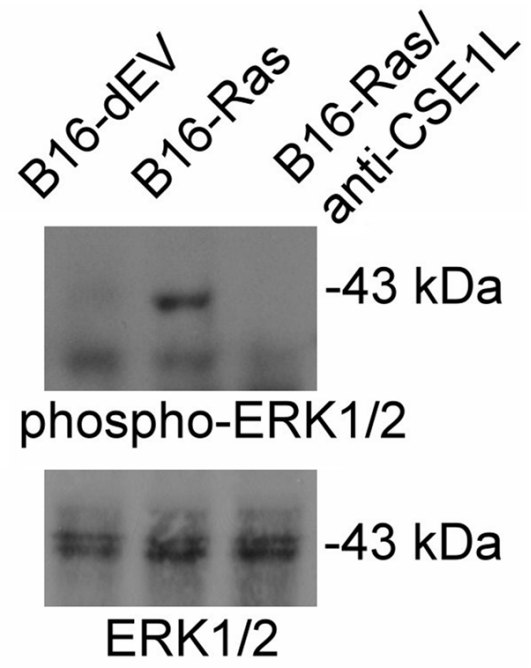

Figure 5. Ras-induced ERK1/2 phosphorylation is regulated by CSE1L. The levels of phospho-ERK1/2 and ERK1/2 expression in B16-dEV, B16-Ras, and B16-Ras/anti-CSE1L cells were analyzed by immunoblotting with antibodies against phospho-ERK1/2 or ERK1/2 as indicated. Each immunoblot was repeated at least three times and showed similar results. The data shown here are the representative immunoblots. Note that CSE1L knockdown inhibited ERK1/2 phosphorylation induced by Ras.

The expression and distribution of phosphor-CSE1L in human melanoma was studied by immunohistochemistry. The results showed that all melanomas (100%, 34/34) exhibited significant positive phospho-CSE1L staining and the tumor cells showed strong cytoplasmic phosphor-CSE1L staining, while nevus cells showed weak cytoplasmic phosphor-CSE1L staining (**Figure 6**).

Discussion

Melanomas are easy to metastasize and difficult to treat. Approximately 60% of patients who die from melanoma have gastrointestinal (GI) metastases [26]. Melanoma metastasis to the lungs is not uncommon and carries a poor prognosis [27]. Our previous study showed the high expression of cytoplasmic phosphorylated CSE1L in malignant melanoma but not in benign nevi [28]. With the use of antibody against phosphorylated CSE1L, the present studies showed that *Ras* mutation in melanoma cells increased CSE1L phosphorylation, phosphorylated CSE1L is mainly distributed in the cytoplasm of melanoma cells as compared with non-phosphorylated CSE1L, and CSE1L knockdown decreased *Ras*-induced phosphorylation of ERK1/2. These results highlight a role of phosphorylated CSE1L in modulating the signaling cascade pathway of melanoma harboring *Ras* mutation, and indicated that CSE1L is a potential target for the control of melanoma malignant progression of induced by Ras.

The Ras family consists mainly of three functional genes (*K-Ras*, *H-Ras*, and *N-Ras*) and the three forms of Ras are preferentially detected in certain tumor types [29]. However, the three *Ras* genes produce the same phenotype in *in vitro* transformation assays [29]. The study used *v-H-Ras* but not *N-Ras*; nevertheless, *v-H-Ras* expression induced the phosphorylation of ERK1/2 and CSE1L of melanoma cells. These results indicated a link between Ras/ERK signaling and phospho-CSE1L in the progression of melanoma.

CSE1L is both a cytoplasmic and a nuclear protein. Nuclear CSE1L has been reported to regulate the transcriptional activity of p53 protein [30]. CSE1L is also named as exportin-2 that mediates importin-α re-export from the nucleus to the cytoplasm after import substrates have been released into the nuclei of cells [31]. The present results showed that phosphorylated CSE1L was mainly distributed in the cytoplasm of cells as compared with non-phosphorylated CSE1L (**Figures 3** and **4**). This result suggests that the phosphorylation status of CSE1L may be involved in the importin-α re-export process.

CSE1L has been reported to be involved in the generation of microvesicles induced by Ras in tumor cells [18]. Tumor-derived microvesicles are rich in metastasis-related proteases and play essential pathological roles in the metastasis of cancer [32, 33]. The phosphorylated CSE1L was mainly distributed in the cytoplasm of melanoma cells and Ras overexpression increased the presence of microvesicles on the membranes of B16F10 melanoma cells (**Figure 4**). Moreover, the staining of phosphorylated CSE1L in microvesicles was relatively higher than that of CSE1L (**Figures 3** and **4**). Microvesicles/exosomes play important role in cancer metastasis [34, 35]. Our results suggest

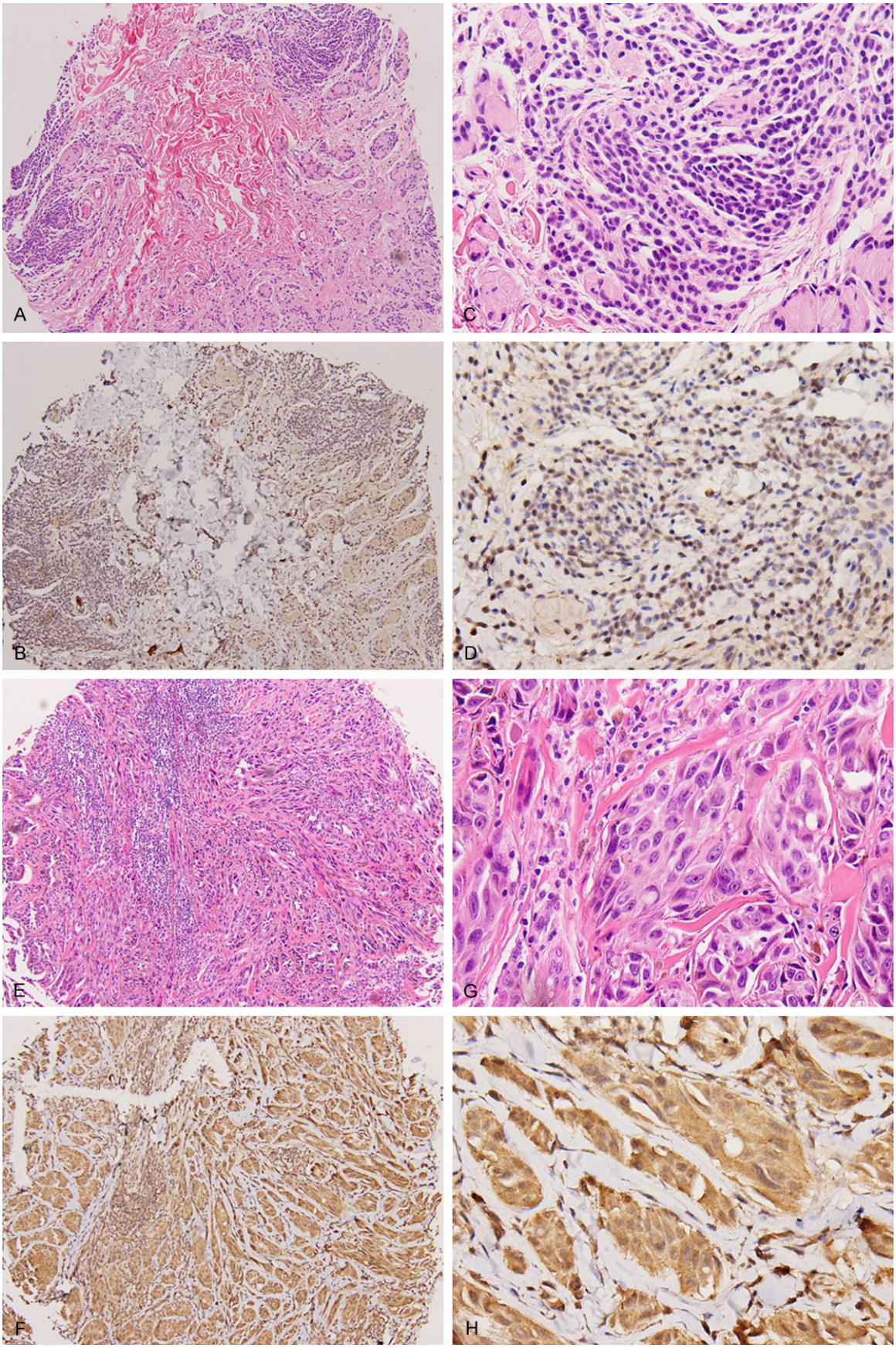
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Figure 6. Representative immunohistochemical images of phosphor-CSE1L expression in human nevi (A-D) and melanomas (E-H). (A, C, E, G) Hematoxylin and eosin staining. (B, D, F, H) Immunohistochemical staining with anti-phosphor-CSE1L antibodies. Original magnification: (A, B, E, F) × 100; (C, D, G, H) × 400.

that the phosphorylation status of CSE1L may be involved in the metastasis of melanoma cells.

Our studies showed that the phosphorylation of CSE1L is involved in the signaling transduction cascade of melanoma cells induced by the *Ras* oncogene. Ras regulates the cytoplasmic distribution of phosphorylated CSE1L; CSE1L knockdown decreased the phosphorylation of ERK1/2 induced by Ras. Thus, phosphorylatied CSE1L may be a potential target for the treatment of melanoma harboring Ras mutation.

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Disclosure of conflict of interest

None.

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