CSE1L modulates Ras-induced cancer cell invasion: correlation of K-Ras mutation and CSE1L expression in colorectal cancer progression

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KEYWORDS: Colorectal cancer; CSE1L; K-Ras; Lymph node; Metastasis

Abstract

BACKGROUND: Ras plays an important role in colorectal cancer progression. CSE1L (chromosome segregation 1-like) gene maps to 20q13, a chromosomal region that correlates with colorectal cancer development. We investigated the association of CSE1L with Ras in colorectal cancer progression.

METHODS: The effect of CSE1L on metastasis-stimulating activity of Ras was studied in an animal model with tumor cells expressing CSE1L-specific shRNA and v-H-Ras. CSE1L expression was evaluated by the immunohistochemical analysis of 127 surgically resected colorectal tumors. K-Ras mutations were analyzed by direct sequencing.

RESULTS: CSE1L knockdown reduced Ras-induced metastasis of B16F10 melanoma cells in C57BL/6 mice. v-H-Ras expression altered the cellular trafficking of CSE1L and increased CSE1L secretion. Most colorectal tumors were positive for CSE1L staining (98.4%, 125 of 127). Colorectal tumors with K-Ras mutation or high cytoplasmic CSE1L expression were correlated with T status (depth of tumor penetration; \( P = .004 \)), stage (\( P = .004 \)), and lymph node metastasis (\( P = .019 \)).
Colorectal cancer is one of the most frequently diagnosed malignancies, and it results in high mortality globally. It exhibits a high frequency of recurrence and metastasis, posing challenges in disease prognosis and the selection of therapeutic treatments. The Ras signaling pathway plays an essential role in the development and progression of colorectal cancer.\(^1\)–\(^3\) In colorectal adenocarcinoma, the K-Ras gene is the predominantly mutated Ras gene, and codons 12, 13, 61, and 146 are hot spots for mutations in K-Ras.\(^4\)\(^,\)\(^5\)

In addition to activation by gene mutation, it has also been reported that K-Ras signaling can be activated through certain stimuli, such as hypoxia, in the absence of a mutation.\(^6\)

Chromosome segregation 1-like protein (CSE1L) is the human homologue of CSE1, the yeast chromosome segregation protein.\(^7\) CSE1L is highly expressed in most cancers, and its expression is correlated with advanced cancer grade and stage.\(^8\)–\(^16\) Recent studies have shown that CSE1L is a secretory protein and is implicated in cancer invasion and metastasis.\(^17\)–\(^21\) CSE1L is located on the 20q13 chromosomal region, a region that frequently harbors amplifications that correlate with the development of colorectal cancer.\(^11\),\(^22\),\(^23\)

Because both Ras and CSE1L have been implicated in colorectal tumor development, an understanding of the relationship between Ras and CSE1L in colorectal cancer progression is needed. This study examined the association of Ras and CSE1L in colorectal cancer progression. The study results indicate that CSE1L is associated with Ras in the malignant progression of tumor cells. Furthermore, K-Ras mutations and CSE1L expression correlated with the clinical manifestations of colorectal cancer, including the cancer stage, T status (depth of tumor penetration), and the occurrence of lymph node metastasis.

**Methods**

**Cells and DNA transfections**

B16F10 melanoma cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured as described elsewhere.\(^18\) Using the Lipofectamine plus reagent (Invitrogen, Carlsbad, CA), cells were transfected with (1) the control pZIP-NeoSV(X)1 vector\(^22\) and the control shRNA plasmids (sc-108060; Santa Cruz Biotechnology, Santa Cruz, CA); (2) the pZIP-v-H-Ras plasmids\(^24\) and the control shRNA plasmids; and (3) the pZIP-v-H-Ras and the CSE1L shRNA plasmids (sc-29909-SH; Santa Cruz Biotechnology) to obtain the B16-dev, B16-Ras, and B16-Ras/anti-CSE1L cell lines, respectively. Transfected cells were selected for 3 weeks with 1 mg/mL G418, followed by selection with 1 μg/mL puromycin for 3 weeks. Multiple drug-resistant colonies (>50) were pooled and propagated as a single culture. The transfected cells were maintained in media containing 200 μg/mL G418 and 0.2 μg/mL puromycin; for the experiments, cells were cultured in medium without G418 and puromycin.

**Conditioned medium**

Cells were grown to subconfluence, washed with phosphate-buffered saline (PBS), and changed to medium without fetal bovine serum. After incubation for 24 hours, the conditioned medium was collected and the cell numbers were counted. To remove possible suspended cells or cell debris, medium was centrifuged at 10,000 rpm for 10 minutes, after which supernatant was harvested. In experiments where cells were treated with DMSO or PD98059, the relative volume of solvent to total media was 5:10,000. The level of CSE1L in conditioned media is very low and is hard to be detected. For analyzing CSE1L level, each conditioned media (above 70 μl) was subjected to immunoblotting, the samples were reacted with anti-CSE1L antibodies at 4 °C for overnight, and the bands were detected with enhanced chemiluminescence method.

**Immunoblotting**

Immunoblotting was performed using anti-p21/ras (EP1125Y; Epitomics, Burlingame, CA) or anti-CSE1L (clone 24; Santa Cruz Biotechnology) monoclonal antibodies, as described elsewhere.\(^18\) The biotin-conjugated anti-CSE1L antibody was prepared using the Biotin Labeling Kit-NH2 kit (Dojindo Laboratories, Kumamoto, Japan). Horseradish peroxidase-conjugated streptavidin and the luminata Forte Western HRP Substrate (Millipore, Billerica, MA) were used to visualize the primary antibody that bound to the protein in the blots.

**Immunofluorescence**

Cells grown on glass cover slides (12 × 12 mm) for 4 days were changed to medium without fetal bovine serum. After incubation for 24 hours, the cells were cytospun at 1,000 rpm for 10 minutes. Cells were washed with PBS, fixed with 4% paraformaldehyde, permeabilized with methanol, and blocked with PBS containing 0.1% bovine serum albumin. Samples were incubated with anti-CSE1L or MMP-2 (H-76, Santa Cruz Biotechnology) for 1 hour. Samples were then washed 3 times with PBS and followed by incubation with secondary antibodies coupled to Alexa.
Fluor 488 (or 568) (Molecular Probes, Eugene, OR) and examined with an inverted fluorescence microscope.

**In vitro invasion assay**

The Matrigel-based invasion assay was performed using Matrigel (BD Biosciences, San Jose, CA) and 8 µm pore, polyvinylpyrrolidone-free polycarbonate filters (Costar, Cambridge, MA), as described elsewhere. The filters were soaked in Matrigel diluted 5-fold with the DMEM medium before use. Triplicate assays were performed, and each assay consisted of 4 replicates. For each replicate, the average number of migrated tumor cells in 10 randomly selected fields was determined.

**Animal metastasis model**

All procedures adhered to the guidelines of the Animal Care Committee of Academia Sinica, Taiwan. C57BL/6 mice aged 6 to 7 weeks (National Laboratory Animal Center, Taipei, Taiwan) were housed in an animal holding room at 22°C with 50% humidity and 12-hour light–dark cycles. Each mouse was injected in the tail vein with 3 × 10^5 viable cells in 100 µL PBS. The B16-dEV, B16-Ras, and B16-Ras/anti-CSE1L cells were injected into 11, 8, and 11 mice, respectively. The mice were sacrificed at 3 weeks’ postinjection and necropsied. The number of lung tumors in each mouse was counted using macrography and micrography.

**Patients**

This study was approved by the ethics committee of the Taipei Medical University Hospital, Taipei, Taiwan. All study participants provided prior informed written consent. Colorectal cancer samples were obtained from 127 patients. There were 75 male and 52 female participants. The mean age was 64.3 years, and the age range was 28 to 93 years. The tumors were graded and categorized according to the 7th edition of the TNM Classification of Malignant Tumours.

**K-Ras gene mutation analysis**

The tumor specimens were immediately frozen following surgical resection and stored in liquid nitrogen. DNA extraction was performed as described elsewhere. The oligonucleotide primers used for amplifying the exon–intron junctions and the coding regions of exons 2, 3, and 4 of the K-Ras gene were 5'-ACACGTTCAGTCACCTGG-3' and 5'-TAACTGGACCCACTAGT-3' for exon 2 (codon 1 to 37); 5'-GCACGTGAAATAACCCAGCT-3' and 5'-CATGGAGGTCACACATTCT-3' for exon 3 (codon 38 to 97); and 5'-GACAAAGTTGGACAGGT-3' and 5'-TAGCATATTGAGAAAACACT-3' for exon 4 (codon 98 to 150). PCR was performed with a denaturing step at 94°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 60 seconds, with a final extension step at 72°C for 5 minutes. All the mutations in K-Ras codons 12, 13, 61, and 146 were confirmed by sequencing both DNA strands of the PCR products on a Beckman Coulter CEQ 8000 Series Genetic Analysis System (Beckman Coulter, Fullerton, CA) using the same oligonucleotide primers that were used to amplify the exon DNA in which the codon was contained.

**Immunohistochemical tissue microarray**

Core biopsies from colorectal carcinoma tumors and noncancerous tissues from colorectal glands were embedded in paraffin blocks, longitudinally cut, and arranged into new paraffin blocks using a manual method of the BiosynMatric Handmade Kit (Formosa Transcrip, Kaohsiung, Taiwan) to generate tissue microarrays. Tissue sections (4 µm) were stained with hematoxylin and eosin to confirm the presence of the morphologically relevant areas of the original cancers. Immunohistochemistry was performed using a 100-fold dilution of an anti-CSE1L monoclonal antibody (clone 3D8, Abnova, Taipei, Taiwan), as described elsewhere.

**Semiquantitative scoring system**

Each tumor was given a score according to the percentage of immunoreactive cells (quantity score) and the intensity (staining intensity score) of the nuclear or cytoplasmic staining, with the rating being confirmed by 2 qualified pathologists. Immunoreactivity was determined by multiplying the staining intensity and the percentage of stained cells, with a minimum possible score of 0 and a maximum of 300. We defined a score of 200 or higher as highly positive. We subdivided the CSE1L immunohistochemical staining into high-CSE1L expression (2+ and 3+) and low-CSE1L expression (0 and 1+) subgroups.

**Statistical analysis**

Significant differences in the clinical and pathological variables between each group were evaluated using the Fisher exact test. Statistical analysis was performed using the Statistical Package for Social Sciences, version 15.0 (SPSS, Chicago, IL), and a probability of <.05 (2-tailed test) was considered statistically significant.

**Results**

Aberrant activation of Ras has been implicated in the metastasis of tumors. We conducted animal experiments to study the relationship between CSE1L and Ras in cancer development. The B16F10 melanoma cell line is widely used as a model for studying numerous aspects of cancer biology, including metastasis, and the cells have been shown to be highly metastatic in C57BL/6 mice. B16F10 cells were transfected
with the control vector, v-H-Ras expressing vector, and v-H-Ras plus CSE1L-specific shRNA expressing vector to obtain the B16-dEV, B16-Ras, and B16-Ras/anti-CSE1L cells, respectively (Fig. 1A). We analyzed the secretion of CSE1L with the cell number standardized conditioned media harvested from serum-starved B16-dEV and B16-Ras cells treated with DMSO or 50 umol/L PD98059 were analyzed by immunoblotting with biotin-conjugated anti-CSE1L antibodies and horseradish peroxidase-conjugated streptavidin. α-tubulin levels were assayed as a control. Each 25 μg of cell lysate and about 70 μl of each conditioned media was analyzed for CSE1L level. The band intensities of secretory CSE1L in immunoblots were quantified by densitometry, and the fold relative to the control is shown. Each immunoblot analysis was repeated at least 3 times, shown here are representative immunoblots.

Figure 1  v-H-Ras expression increases CSE1L secretion. (A) Western blotting analysis of Ras and CSE1L expression in B16-dEV, B16-Ras, and B16-Ras/anti-CSE1L cells with anti-CSE1L (clone 24) and anti-Ras antibodies. β-actin levels were assayed as a control. (B) Levels of CSE1L in the cell lysates and the cell number-standardized conditioned media harvested from serum-starved B16-dEV and B16-Ras cells treated with DMSO or 50 umol/L PD98059 were analyzed by immunoblotting with biotin-conjugated anti-CSE1L antibodies and horseradish peroxidase-conjugated streptavidin. α-tubulin levels were assayed as a control. Each 25 μg of cell lysate and about 70 μl of each conditioned media was analyzed for CSE1L level. The band intensities of secretory CSE1L in immunoblots were quantified by densitometry, and the fold relative to the control is shown. Each immunoblot analysis was repeated at least 3 times, shown here are representative immunoblots.

with the control vector, v-H-Ras expressing vector, and v-H-Ras plus CSE1L-specific shRNA expressing vector to obtain the B16-dEV, B16-Ras, and B16-Ras/anti-CSE1L cells, respectively (Fig. 1A). We analyzed the secretion of CSE1L with the cell number standardized conditioned media harvested from serum-starved B16-dEV and B16-Ras cells treated with DMSO or 50 umol/L PD98059, a potent extracellular signal-regulated kinase activity inhibitor. The result of immunoblotting showed that the level of CSE1L was higher in the conditioned medium harvested from B16-Ras cells than that in the conditioned medium harvested from B16-dEV cells, and PD98059 treatment attenuated the v-H-Ras-increased CSE1L secretion; although v-H-Ras expression did not increase CSE1L expression in cells and PD98059 treatment did not decrease CSE1L expression in cells (Fig. 1B). Thus, v-H-Ras expression stimulated CSE1L secretion in an extracellular signal-regulated kinase dependent manner. CSE1L is located in both the cytoplasm and the nuclei of cancer cells, and cytoplasmic CSE1L is implicated in cancer invasion and metastasis. The B16F10 cancer cell line is a highly metastatic cancer cell line, and immunofluorescence showed that CSE1L was located mainly in the cytoplasm of B16F10 cells (Fig. 2). Moreover, the results of immunofluorescence showed that v-H-Ras transfection stimulated the accumulation of CSE1L in cytoplasmic areas near the membrane of B16-Ras cells (Fig. 2). It is also interesting to note that matrix metalloproteinase-2 (MMP-2), an extracellular protease that plays an important role in tumor invasion and metastasis, also accumulated in cytoplasmic areas near the membrane of B16-Ras cells and was colocalized with CSE1L (Fig. 2). Thus, v-H-Ras expression altered the cellular trafficking of CSE1L and MMP-2. CSE1L is a secretory protein and it is implicated in cancer invasion and metastasis. The Matrigel-based invasion assay showed that v-H-Ras expression increased the in vitro invasion activity of B16-F10 cells, and that CSE1L knockdown attenuated this response (Fig. 3A). The animal model showed that v-H-Ras expression increased the pulmonary metastasis of the tumor cells by 246.1% (P < .03), and that CSE1L knockdown attenuated the v-H-Ras-induced pulmonary metastasis by 100% (P < .01) (Fig. 3B); the growth rates of the B16-Ras and B16-Ras/anti-CSE1L cells were similar (Fig. 3C). These results indicate that CSE1L mediates the invasion- and metastasis-stimulating activities of Ras.

There were 20 patients with stage I tumors, 47 patients with stage II tumors, 44 patients with stage III tumors, and 16 patients with stage IV tumors in the study. Of these tumors, 4 were low grade, 116 were moderate grade, and 7 were high grade. The results of the K-Ras mutation analysis of human colorectal carcinoma tumors showed that K-Ras mutation occurred in 32.2% (41 of 127) of the cases. The mutational frequencies of codons 12, 13, and 146 were 22.2%, 9.5%, and 0.8%, respectively. Of the 41 K-Ras mutation cases, 28 cases were codon 12 mutations, 12 cases were codon 13 mutations, and 1 case was a codon 146 mutation. All were heterozygous mutations.
No mutations in codon 61 were identified. In the immuno-
histochemical analysis of the colorectal tumors and the 
marginal normal tissues, the nonneoplastic colorectal tis-
sues were weakly stained using the anti-CSE1L antibody. 
However, 98.4% (125 of 127) of the colorectal carcinoma 
biopsies exhibited significantly darker staining. These re-
results indicate that immunohistochemical analysis of 
CSE1L expression may be a valuable diagnostic tool for 
colorectal cancer.

Our in vitro invasion and animal metastasis studies 
showed that K-Ras was associated with CSE1L in regulating 
the progression of tumor. Thus, we investigated the clinical 
and pathological correlation of K-Ras and CSE1L in colo-
rectal cancer. The CSE1L immunohistological staining in 
neoplastic colorectal glands was subdivided into high and 
low CSE1L staining subgroups (Fig. 4). For cytoplasmic 
staining, 49.6% of the cases were classified as low CSE1L 
staining (63 of 127), and 51.1% of the cases showed high 
CSE1L staining (65 of 127). For nuclear staining, 55.1% 
of the cases were classified as low CSE1L staining (70 of 
127), and 45.6% of the cases showed high CSE1L staining 
(58 of 127). The association of the K-Ras mutations with 
the CSE1L immunohistochemical staining and the clinical 
parameters is summarized in Tables 1 and 2. Statistical anal-
ysis showed that the occurrence of either the K-Ras muta-
tions or the high cytoplasmic CSE1L staining in tumors 
was associated with an advanced cancer stage, high T status, 
and the occurrence of lymph node metastasis in colorectal 
cancer (Table 1). No statistical significance was noted be-
tween tumors with K-Ras mutation or nuclear high CSE1L 
staining and the clinical manifestations (Table 2). These re-
results indicate that mutations in K-Ras gene and cytoplasmic 
expression of CSE1L protein are correlated with the clinical 
and pathological features of colorectal cancer.

Comments

In addition to promoting tumor initiation, the Ras onco-
gene also plays a significant role in the malignant

![Figure 2](image_url)
progression of tumors. Activation of Ras is correlated with lymph node metastasis and tumor stage in colorectal cancer. The CSE1L gene is located in the 20q13 region of chromosome 20. Amplifications in the 20q13 chromosomal region correlate with colorectal cancer development. Thus, the roles of Ras and CSE1L in the progression of colorectal cancer may be related. We showed that v-H-Ras expression stimulated CSE1L secretion, and reduced CSE1L expression attenuated the v-H-Ras-induced invasion and metastasis of B16F10 cancer cells (Figs. 1 and 3). Enhanced CSE1L expression has been shown to increase the invasion and metastasis abilities of cancer cells. The present results showed that CSE1L knockdown reduced Ras-induced invasion and metastasis of tumor cells (Fig. 3). These results indicate that CSE1L is associated with Ras in cancer progression through its mediation of the Ras-triggered invasion and metastasis. Thus, CSE1L may represent a therapeutic target for inhibiting the malignant progression of colorectal cancer.

Our results also showed that colorectal cancer with K-Ras mutation or high cytoplasmic CSE1L expression was correlated with an advanced cancer stage, high T status, and the occurrence of lymph node metastasis. There was no statistical significance when analyzing the patient data with and without K-Ras mutation and with low vs high CSE1L expression without analyzing CSE1L nuclear versus cytoplasmic expression (data not shown). Also, no statistical significance was found when analyzing the patient data with K-Ras mutation and either cytoplasmic or nuclear CSE1L expression (data not shown). K-Ras mutation rates of 20% to 50% have been reported for colorectal cancer. Kwon et al. reported a 20.7% mutation rate for K-Ras in advanced colorectal cancer.
Figure 4  Representative immunohistochemical images of CSE1L expression in the nontumor colorectal glands and the colorectal carcinomas. (A, B) Nonneoplastic colorectal glands showed faint staining of the CSE1L protein. (C–F) Cytoplasmic CSE1L expression in the colorectal carcinoma tumor cells that were classified as low- (C, D) and high-CSE1L staining (E, F). Nuclear CSE1L expression in the colorectal carcinoma tumor cells (G–J) that were classified as low- (G, H) and high-CSE1L staining (I, J). Original magnification: A, C, E, G, and I ×100; B, D, F, H, and J ×400.
Our data showed a $K$-Ras mutation frequency of 32.2%. The low $K$-Ras mutation frequency in our data may have contributed to the absence of a significant correlation between the $K$-Ras mutations and the clinical manifestations of colorectal cancer in the results of our statistical analysis.

In the absence of a mutant $K$-Ras, the activation of Ras signaling in response to hypoxia has been reported, suggesting that the downstream mechanisms involved in the activation of Ras can be influenced by nongenetic factors. Therefore, although the mutation of $K$-Ras is not very common in colorectal cancer, the downstream signaling of Ras can nonetheless play an essential role in the malignant progression of colorectal cancer. We showed that CSE1L mediates Ras-triggered invasion and metastasis of cancer cells, and that most colorectal carcinomas were significantly positive for CSE1L staining. Thus, CSE1L may be a useful immunohistochemical marker for colorectal cancer diagnosis.

**Table 1** Correlation of colorectal tumors with $K$-Ras mutations or high cytoplasmic CSE1L expression

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<tr>
<td>T3+T4</td>
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<tr>
<td>II/III/IV</td>
<td>29 (27.1)</td>
<td>78 (72.9)</td>
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*Data are shown as number of cases (%).
†Statistically significant.

**Table 2** Correlation of colorectal tumors with $K$-Ras mutations or high nuclear CSE1L expression

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<td>T3+T4</td>
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*No. of cases (%).
Our data showed that CSE1L knockdown in B16-Ras cells resulted in a reduced metastasis activity of the cells (ie, the B16-Ras/anti-CSE1L cells) compared with the B16-dEV control cells (Fig. 3). B16-dEV cells expressed a certain level of CSE1L (Fig. 1A). This result indicates that CSE1L plays an important role in cancer invasion and metastasis. For example, CSE1L was reported to regulate microtubule assembly, stimulate invadopodia extension, and increase the migration of tumor cells.\(^4\) Also, CSE1L was reported to regulate MMP-2 secretion and increase the invasion and metastasis of tumor cells.\(^3\) We cannot exclude the possibility that at least to some extent, other cellular metastasis-stimulating factors also regulate cancer metastasis by modulating CSE1L.

CSE1L is located in both the cytoplasm and nucleus of cells. Nuclear CSE1L regulates the transcriptional activity of the p53 protein and the transport of proteins into the nucleus.\(^4\)\(^,\)\(^4\)\(^,\) As mentioned above, cytoplasmic CSE1L is associated with microtubules, and the association of CSE1L with microtubules stimulates the extension of invadopodia and enhances the migration activity of tumor cells.\(^3\) Microvesicles are cytoplasmic membrane–derived vesicles that are released from cells by the outward budding and fission of the cytoplasmic membrane.\(^4\)\(^2\) Tumor-derived microvesicles are rich in metastasis-related proteases and play roles in the interactions between tumor cells and tumor microenvironment in tumor metastasis.\(^4\)\(^3\) It is interesting to note that CSE1L was recently reported to modulate Ras-induced microvesicle generation of tumor cells.\(^3\)\(^4\) These findings indicate that cytoplasmic CSE1L may play an important role in metastasis and suggest that cytoplasmic CSE1L may be a prognostic marker for cancer.

Conclusions

Accurate classification of the T status and the tumor stage is essential for determining colorectal cancer treatment options. Lymph node metastasis is a particularly important prognostic indicator for disease progression and is crucial for the determination of therapeutic strategies for colorectal cancer.\(^4\)\(^,\)\(^6\) Our data showed that colorectal cancer with K-Ras mutation or high cytoplasmic CSE1L expression correlates with advanced cancer stage, T status, and the occurrence of lymph node metastasis (Tables 1 and 2). A relationship between CSE1L expression and lymph node metastasis in colorectal cancer has been reported recently.\(^4\)\(^7\) Collectively, these findings suggest that CSE1L may be a valuable immunohistochemical marker for colorectal cancer prognosis when used in combination with K-Ras mutation analysis.

References

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