RESEARCH ARTICLE

Suppression of Cellular Apoptosis Susceptibility (CSE1L) Inhibits Proliferation and Induces Apoptosis in Colorectal Cancer Cells

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Abstract

The cellular apoptosis susceptibility (CSE1L) gene has been demonstrated to regulate multiple cellular mechanisms including the mitotic spindle check point as well as proliferation and apoptosis. However, the importance of CSE1L in human colon cancer is largely unknown. In the present study, we examined expression levels of CSE1L mRNA by semiquantitative RT-PCR. A lentivirus-mediated small interfering RNA (siRNA) was used to knock down CSE1L expression in the human colon cancer cell line RKO. Changes in CSE1L target gene expression were determined by RT-PCR. Cell proliferation was examined by a high content screening assay. *In vitro* tumorigenesis was measured by colony-formation assay. Cell cycle distribution and apoptosis were detected by flow cytometric analysis. We found CSE1L mRNA to be expressed in human colon cancer cells. Using a lentivirus based RNAi approach, CSE1L expression was significantly inhibited in RKO cells, causing cell cycle arrest in the G2/M and S phases and a delay in cell proliferation, as well as induction of apoptosis and an inhibition of colony growth capacity. Collectively, the results suggest that silencing of CSE1L may be a potential therapeutic approach for colon cancer.

Keywords: CSE1L - colorectal cancer - apoptosis - proliferation

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Introduction

Colorectal cancer or colon cancer is one of major public health diseases (O'Brien et al., 2007). It was estimated around one million cases of colon cancer are diagnosed per year (Chintharlapalli et al., 2011). Although several therapies such as surgery, ablation, chemotherapy and chryotherapy continue to emerge, yet it remains the second cause of cancer related death. Efforts have been made to search for molecular markers and therapeutic targets to improve the early diagnosis and prognosis of colorectal patients. Several candidate genes such as VEGF (Kim et al., 2008), GATA-4 and GATA-5 (Akiyama et al., 2003), SFRP (Suzuki et al., 2004), CSE-1L and COX-2 (Eberhart et al., 1994) have been implicated in disease progression and new therapeutic approaches against these targets have potential for the treatment of colon cancers. CSE1L is the human homologue to the yeast gene CSE1. CSE1L gene is implicated in the regulation of multiple cellular mechanisms including the mitotic spindle check point as well as in proliferation and apoptosis, two opposing cellular mechanisms which are tightly linked in mammalian cells (Behrens et al., 2003). CSE1L is located on chromosome 20q13, a locus amplified for example in colon cell-lines and in 20–30% of breast cancers (Behrens et al., 2003). Pathological studies demonstrated that CSE1L was highly expressed in various cancers, and the expression of CSE1L was positively correlated with high cancer stage, high cancer grade and worse outcome in cancer patients (Tsao et al., 2009b). Several studies have reported that CSE1L regulates the secretion of human colorectal cancer cells, and metastasis of several cancer such as prostate and breast cancer cells (Ching-Fong et al., 2008; Tsao et al., 2009b; Tung et al., 2009). Moreover, CSE1L also facilitates the migration of breast cancer cell lines (Tai et al., 2010). Therefore, CSE1L may present a potential molecular target in colon cancer therapy

RNA silencing or as known as RNA interferences (RNAi) has been demonstrated to be highly effective and promising in the field of cancer therapy (Castanotto and Rossi, 2009). The two main strategies are delivery of chemically synthesized RNAi (non-viral delivery), or delivery of short hairpin RNA (shRNA)-encoding genes by engineered viruses that will ultimately generate RNAi by transcription in the target cells (Pai et al., 2005). Up to now, many gene products involved in carcinogenesis

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have already been explored as targets for RNAi. In most studies, the silencing of critical gene products by RNAi technology has generated significant antiproliferative and/or proapoptotic effects in cell-culture systems or in preclinical animal models (Castanotto and Rossi, 2009). Moreover, the advantage of RNAi technology is that it can be used to target a large number of different genes involving a number of distinct cellular pathways (Zhang et al., 2007). This is particularly important for a disease as complex as cancer.

In the present study, we used lentiviral vector-mediated delivery specific shRNA against CSE1L to knock down the expression of CSE1L in human colon cancer (RKO) cell line. Furthermore, we also investigated the effect of CSE1L down regulation on cell proliferation and cell cycle progression and to be probe the feasibility of gene therapy for colon cancer.

Materials and Methods

Reagents and antibodies

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), TRIzol reagent and Lipofectamine 2000 were obtained from Invitrogen (Carlsbad, USA); Giemsa staining was obtained from Chemicon International (Temecula, CA, USA); Propidium Iodide (PI) was obtained from keyGEN biology (Nanjing, China); M-MLV Reverse Transcription was obtained from Promega (Madison, WI, USA); oligo-dT was obtained from Sangon Biotech (Shanghai, China); SYBR green master mixture was obtained from Takara (Otsu, Japan); pGCL-GFP vector and virion packaging elements (pHelper 1.0 and pHelper 2.0) were obtained from Genechem (Shanghai, China). Other chemical and reagents used in this study were of analytical grade.

Cell culture and cell survival assay

Five colorectal cancer cell lines (HT116, RKO, SW480, SW620 and LoVo) were obtained from the American Type Culture Collection (Manassas, VA). All cell lines were maintained in appropriate media containing 10% fetal bovine serum at 5% CO_2 and 37°C. The cell numbers was assessed by Cellomics Arrayscan VTI HCS reader (Thermo Scientific, Waltham, MA, USA). The results were normalized with the day one, considering the cell counts value in day one is one. The cell number was counted each day for successive 5 days after plating. Each assay was repeated at least three times.

Preparation of RNAi constructs and transfection.

RNAi target sequence to human CSE1L (5'-GCATGGAATTACAAAGCAAA-3') was designed by GeneChem, and the oligonucleotides encoding the CSE1L short hairpin RNA (with a loop sequence separating the complementary domains) were synthesized. The forward strand is 5'-CCGGGCATGGAATTACACAAGCAAATTCAAGAGATTTGCTTGTGTAATTCCATGCTTTTTG -3', and the reverse strand is 5'-AATTCAAAAAGCATGGAATTACACAAGCAAATCT CTTGAATTTGCTTGTGTAATTCCATGC -3'. The negative control siRNA sequence is 5'-TTCTCCGAACGTGTCACGT-3'. These shRNA sequences were inserted into the lentiviral vector. The recombined plasmid and virion packaging elements were then cotransfected into HEK293 cells using Lipofectamine 2000™ according to the manufacturer's instruction. At 48 h after transfection, the culture medium containing the viral particle was collected, centrifuged at 4000 g for 10 min and filtered through a 0.45 μm filter. The viral supernatant was further concentrated with a Centricon Plus-20 Centrifugal Filter at 4000 g for 10 min.

RNA extraction and RT-PCR

Total RNA was extracted from RKO cells that transduced with CSE1L-knockdown lentivirus for 72 h by using TRIzol reagent according to the manufacturer's protocol. RNA yield and purity were quantified by UV absorbance spectroscopy. Reverse transcription was performed using MMLV Reverse Transcriptase and oligo (dT) primer. Realtime PCR was conducted with the TAKARA TP800-Thermal Cycler DiceTM Real-Time System using SYBR green Master Mixture. In brief, each PCR reaction mixture containing 10 μl of 2x SYBR Green Master Mix, 1 μl of sense and antisense primers (5 μmol/μl) and 1 μl of cDNA (10 ng), were run for 45 cycles where denaturation at 95 oC for 15 s, annealing at 60 oC for 30 s and extension at 72 oC for 30 s in a total volume of 20 μl. For relative quantification, $2^{-\Delta\Delta CT}$ was calculated and used as an indication of the relative expression levels, which was calculated by subtracting CT values of the control gene from the CT values of CSE1L. The primer sequences for PCR amplification of CSE1L gene were 5'-CGCACCGTTTGTTGAGATTC-3' and 5'-TGATGAGAGTAGGGATGTAGGG-3'. GAPDH was applied as an internal control. The primer sequences of GAPDH were 5'-TGACTTCAACAGCGACACCCA-3' and 5'- CACCCTGTTGCTGTAGCCAAA-3'.

Cell cycle analysis by flow cytometry

Different cell cycle phases (G1, S or G2/M phase) are characterized by different DNA contents. The cells were collected by trypsinization after treatments and fixed with 70% ethanol. Following incubation with RNase (0.1%; Boehringer Mannheim/Roche Diagnostic Corporation, Indianapolis, Ind.), cells were stained with propidium iodide (PI; 50 μg/ml; Roche Diagnostic Corporation), and fluorescence signals from the stained cells were collected in the FL-2 detector using a 585/42 band-pass filter. A total of 100,000 events were collected. The data were analyzed by using Becton Dickinson CELLQuset software.

Apoptotic assay

ApoScreen Annexin V Apoptosis Kit (Southern Biotech, Cat. No. 10010-09) were used for labeling of apoptotic cells. They were performed as follows: after infection with lentivirus carrying CSE1L siRNA or a negative control siRNA, the cells were incubated until the convergence rate reached 75% (5 days after transduction). Then they were trypsinized, centrifuged, then washed by 19 binding buffer and resuspended in 1 ml of Annexin V binding buffer. Then, 5×10^5 cells (100 μl of the cell suspension solution) were stained by 5 μl annexin V-APC following 5 μl PI. For each experiment, 20,000 cells

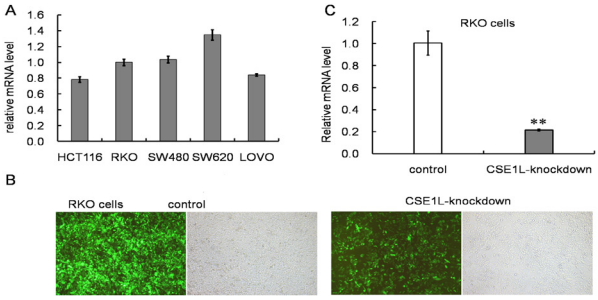

Figure 1. Lentivirus-mediated Knock-down of CSE1L in the Human Colon Cell Line RKO. The expression of CSE1L mRNA in the five human colon cancer cells was measured by Semi-quantitative RT-PCR (a). Transduction efficiency was estimated five days after infection at MOI of 10. Light and fluorescent micrograph of control cells and CSE1L-knockdown cells were shown (×200) (b). Total RNA was extracted five days after infection and relative CSE1L expression was determined by quantitative real time PCR. GAPDH was used as control (c). Data represent the means ± SD of three independent experiments. $^{**}p<0.01$

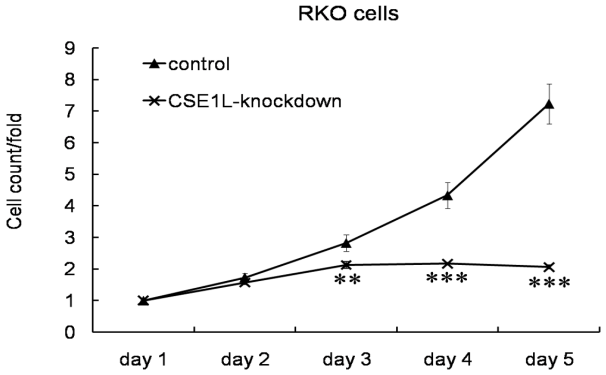

Figure 2. CSE1L-specific shRNA Caused Decreased in RKO Cell Proliferation. The cell number was directly counted using Cellomics Arrayscan VTI HCS reader and the results were normalized with the day one. Data represent the means ± SD of three independent experiments. $^{**}p<0.01$, $^{***}p<0.001$

were analyzed using FACSCalibur flow cytometer (BD Biosciences). Experiments were performed in triplicate.

Colony formation assay

Soft agar assays were performed as follows: briefly, 0.5-ml underlayers consisting of 0.8% agar medium were prepared in six-well plates. Cells infected with CSE1L siRNA-expressing lentivirus or a negative control lentivirus were trypsinized, centrifuged, resuspended in 0.4% agar medium (equal volumes of 0.8% noble agar and culture medium), and plated onto the top agar at 200 cells per well. The cells were cultured for 14 days at 37°C. Colonies were visualized using a cell staining Giemsa solution (Chemicon) and counted under a microscope.

Statistical analysis

The data were presented as means ± SD (n = 3). Differences between the means of the individual groups were assessed by one-way ANOVA with Duncan's multiple range tests. Differences were considered significant at $p < 0.05$. The statistical software package, SPSS v.16 (SPSS Inc., Chicago, Illinois, USA), was used for the analysis.

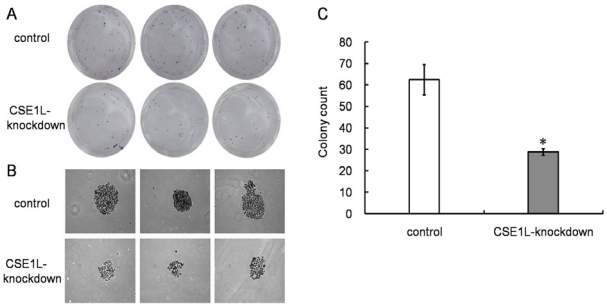

Figure 3. CSE1L-specific shRNA Caused Decreased in Colony Formation in RKO cells. RKO cells were seeded and allowed for 14 days to form colonies. Colony number were counted and recorded (a). The colonies stained with Giemsa were observed under fluorescence microscopy (×200) (b). Statistical analysis showed that CSE1L knocked down RKO cells had fewer colonies compared to the control (c). Data represent the means ± SD of three independent experiments. $^{*}p<0.05$

Results

Downregulation of CSE1L in RKO cells mediated by RNAi

Five human colon cancer cells (HCT116, SW480, SW620, RKO, LoVo) were screened for the presence of CSE1L by assessing CSE1L mRNA levels. Of the five cell lines assayed, the RKO cell line which had the middle expression of CSE1L mRNA was chosen for further experiments (Figure 1A). The RKO cells are poorly differentiated human colorectal adenocarcinoma cells that contain wildtype p53 and have a doubling time of approximately 19.6 hours (Mulkeen et al., 2006).

To investigate the role of CSE1L in human colon cancer, we used lentivirus harboring shRNA against CSE1L to infect RKO cells. The lentivirus infection was successful, as observed by fluorescence microscopy (Figure 1B). We further investigated the effect of lentivirus-mediated silencing of CSE1L expression in RKO cells. Real time PCR analysis revealed that CSE1L gene expression was significantly decreased compared to the control cells (Figure 1C). These results strongly suggest that CSE1L could be knocked down by CSE1L-specific shRNA in RKO cells.

Silencing of CSE1L inhibit RKO cell proliferation

In order to evaluate the effect of CSE1L silencing on cell proliferation, the cell numbers were counted by using Cellomics High-Content Screening machine. Cell count assay indicated significant difference in cell proliferation between controls and CSE1L-knockdown RKO cells. At 3, 4 and 5 days post infection, CSE1L-knockdown RKO cells demonstrated lower cell proliferation compared to control. The number of control cells in the fifth day increase to more than 7 folds compared to CSE1L infected cells ($^{***}p<0.005$). The data were shown in Figure 2. These findings indicated that CSE1L are necessary for RKO cell proliferation.

Silencing of CSE1L inhibit RKO cell growth

The effect of CSE1L knockdown in RKO cell growth was examined. The role of CSE1L in RKO cells were investigated using colony formation of RKO cells. Silencing of CSE1L in RKO cells reduced the colony

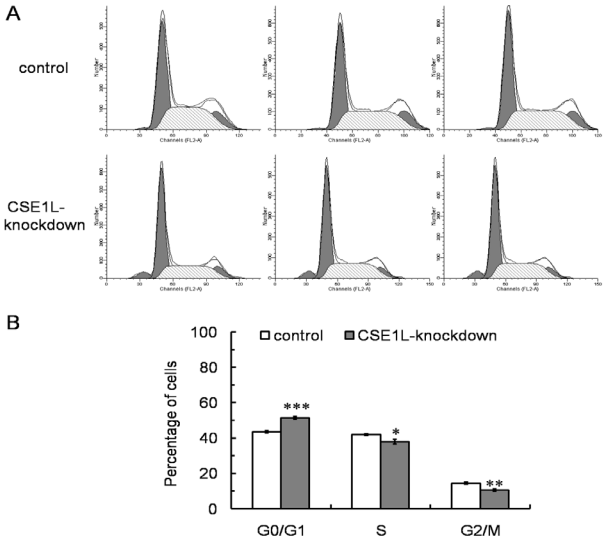

Figure 4. Effect of Down-regulation of CSE1L Expression on Cell Cycle Progression in RKO Cells. Cell cycle distribution was performed by flow cytomeric analysis. Representative graphs (a) and statistical plots (b) of three independent experiments are shown. Data represent the means ± SD of three independent experiments. *p<0.05,

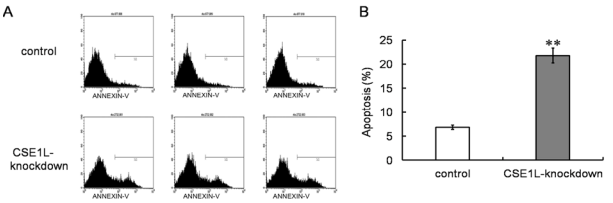

Figure 5. Down-regulation of CSE1L Induces Apoptosis in RKO Cells. Cell apoptosis was determined by Annexin V staining and flow cytometry (a). CSE1L-knockdown cultures showed a significant increase in apoptosis compared with control (b). Data represent the means ± SD of three independent experiments. **p<0.01, compared to control cells

formation in soft agar. As showed in Figure 3A, the number of surviving colonies for CSE1L-knockdown RKO cells was decreased dramatically. Moreover, the cell numbers in each colony were also significantly lower than the control (*p<0.05) (Figure 3B and 3C). It suggests that CSE1L is critical for colorectal tumorigenesis.

Silencing of CSE1L induced cell cycle arrest in RKO cells

Next, to determine whether CSE1L regulates the cell cycle, we examined the cell cycle distribution after infecting RKO cells with CSE1L. The cell cycle progression of RKO cells were analyzed using FACS. As shown in Figure 4, Silencing of CSE1L caused a substantial decrease in the S and G2/M phase and an increase in G1 phase population in RKO cells compared to the control. In the control group the S and G2/M phase accounted for cell percentages of 42.0% and 14.4% respectively. Meanwhile CSE1L knockdown shows cell percentages of 37.9% and 10.6% at S and G2/M phases respectively. This result indicates that more number of cells is being arrested in the G0/G1 phase.

Silencing of CSE1L enhanced apoptosis in RKO cells

In order to confirm whether the silencing of CSE1L reduce the cell survival rates the presence of apoptotic cells with and without CSE1L expression, the FACS using cells stained with Annexin-V were performed. As shown in Figure 5, deletion of CSE1L has increased the percentage of apoptotic cells to 22% compared to control, 7%. This suggest that the CSE1L knocking down gradually leads to apoptosis in RKO cells.

Discussion

Various proteins in humans are not only involved in proliferation and apoptosis but also in cancer development and one of the examples are CSE1L (Brinkmann et al., 1999). CSE1L is a Ran binding protein which, similar to Ran and other Ran binding proteins plays (at the same time) an important role in proliferation and apoptosis. Recent studies demonstrated that CSE1L plays an important role in cancer progression. Tsao et al. (2009) suggest that CSE1L regulates the secretion of colorectal cells and regulate the metastasis of colorectal cancer (Tsao et al., 2009a). More recently, Tai et al. showed that CSE1L plays a role in regulating the extension of cell protrusions and promotes the migration of cancer cells (Tai et al., 2010). However up to now, there are only limited studies related to the effect of CSE1L knockdown in cancer cell lines. Hence, this study was planned to find the effects of CSE1L knockdown in colon cancer cells.

In the present study, we showed that the expression of CSE1L was relatively high in all colon cancer lines tested (HCT116, SW480, SW620, RKO, LoVo). Furthermore, to evaluate the possibility of CSE1L as an effective therapeutic target for colon cancer, we employed lentivirus-mediated shRNA to silence CSE1L expression. Following CSE1L silencing in RKO cells, we found that less number of cells in S and G2/M phases. CSE1L silencing also decreased the colony number in RKO cells. Furthermore, the results of this study reveal, for the first time, the role of CSE1L silencing in RKO cells.

Although the exact mechanisms are not clear, yet we may argue that disturbance of CSE1L in RKO cells, might lead to genetic instability. Unlike other cell-cycle-checkpoint genes involved in cancer development, CSE1L functions in the mitotic spindle checkpoint (Behrens et al., 2001; Bera et al., 2001). Notably, CSE1L is also thought to be responsible for accurate chromosome alignment in the mitotic spindle and accurate chromosome segregation during cell division, which is a hallmark of cancers of various origins (Liao et al., 2008; Wellmann et al., 2001).

In conclusion, the findings of this study indicated that CSE1L exhibit potential therapeutic target of colon. The inhibition of CSE1L expression may considered as therapy approach for the treatment of colon cancer. Based on the result of this study, CSE1L may ultimately prove to be useful in the treatment of colon cancer. Furthermore, future studies are expected to confirm the possible upstream and downstream genes involved in cancer related to CSE1L silencing.

Acknowledgements

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