

Original Article

CSN5 silencing inhibits invasion and arrests cell cycle progression in human colorectal cancer SW480 and LS174T cells *in vitro*

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Abstract: CSN5 has been implicated as a candidate oncogene in human cancers by genetic linkage with activation of the poor-prognosis, wound response gene expression signature. The present study aimed to investigate the effect of silencing CSN5 on invasion and cell cycle progression of human colorectal cancer cells, and to determine the potential molecular mechanisms that are involved. The CSN5 specific small interfering RNA (shRNA) plasmid vector was constructed and then transfected into colorectal cancer cells. The expression of CSN5 mRNA and protein was detected by quantitative polymerase chain reaction and western blot analysis, respectively. Cell adhesion and invasion were analyzed using MTS and Transwell assays, respectively, and cell cycle progression was analyzed using flow cytometry. Adhesion, invasion, and cell cycle distribution were assessed following knockdown of CSN5 by RNA interference (RNAi). Furthermore, knockdown of CSN5 significantly inhibited cell adhesion and reduced the number of invasive cells, while increasing the percentage of cells in the G0/G1 phase ($P < 0.05$). Western blot and real-time PCR analysis were used to identify differentially expressed invasion and cell cycle associated proteins in cells with silenced CSN5. The expression levels of CSN5 in colorectal cancer cells transfected with siRNA were decreased, leading to a significant inhibition of colorectal cancer cell adhesion and invasion. Western blot analysis revealed that silencing of CSN5 may inhibit CD44, matrix metalloproteinase (MMP) 2 and MMP 9 protein expression, significantly promoted cell cycle-related genes P53 and P27 expression. In addition, CSN5 silencing may induce activation PI3K/AKT signal regulated cell invasion. Moreover, CSN5 silencing inhibited the secretion of TGF- β , IL-1 β and IL-6 and the transcriptional activity of transcription factor NF- κ B and Twist in human colorectal cancer cells. Taken together, down regulation of CSN5 may inhibit invasion and arrests cell cycle progression in colorectal cancer via PI3K/AKT/NF- κ B signal pathway, which indicates that there is a potential of targeting CSN5 as a novel gene therapy approach for the treatment of colorectal cancer.

Keywords: CSN5, invasion, colorectal cancer

Introduction

Colorectal cancer, as one of the high-incidence cancers in China, is characterized by its poor overall prognosis, high mortality, and complex pathogenesis process involving joint actions of the genetic factors, tumor micro-milieu and multiple other factors [1, 16]. Accordingly, one of the main contents of current colorectal cancer studies is to thoroughly understand the mechanisms of invasion in order to provide an experimental basis for the diagnosis and treatment of colorectal cancer [17].

CSN5 plays an important role in cell proliferation, differentiation, apoptosis and related

activities [10, 13]. It is considered to be an independent prognostic factor for multiple human tumors and possibly a future target of tumor therapy. CSN5 over-expression is observed in a variety of tumor cells and closely related to tumorigenesis of many tumors. By interacting with a variety of proteins, it not only mediates the signal transduction between the cells and the extracellular matrix but also regulates cell growth, survival, differentiation, proliferation, migration and other cellular processes [9]. Accordingly, this study constructed human colorectal cancer SW480 and LS174T cell lines in which CSN5 expression is silenced to examine the effects of CSN5 on the *in vitro* invasion of tumor cells.

Table 1. Primer sequences for real-time PCR study

Gene	Sequences (5'-3')
CD44	Forward: TGGCACCCGCTATGTCCAG- Reverse: GTAGCAGGGATTCTGTCTG
MMP2	Forward: TGATCTTGACCAGAATACCATCGA Reverse: GGCTTGCAGAGGAAGAAGTT
CDK1	Forward: GGATGTGCTTATGCAGGATTCC Reverse: ATGACTGACCAGGAGGGATAG
MMP9	Forward: TGGGGGGCAACTCGGC Reverse: GGAATGATCTAAGCCAG
α -actin	Forward: TGCCAACAACGTCATGTCG Reverse: CAGCGCGGTGATCTCTTTCT
TP53	Forward: GAGCACTGCCAACAACAC Reverse: GTCTGGTCTGAAGGGTGAA
CSN5	Forward: GCAATCGGGTGGTATCATAGC Reverse: TGCGGATATTGTTCTTGTGGA
GAPDH	Forward: GAAGGTGAAGGTCGGAGTC Reverse: GAAGATGGTGATGGGATTC

Materials and methods

Construction of CSN5 silencing SW480 and LS174T cell lines

Human colorectal cancer SW480 and LS174T cells were cultured in DMEM medium containing 10% fetal bovine serum in an incubator under 37°C and 5% CO₂ condition. Lipofectamine 2000 was used to transfect shRNA into these cells using method described in kit instructions. In 48h after the transfection, medium containing 100 µg/ml G418 was added to carry out stable screening. Once the cells were maintained for 14 d after the drug was added, the monoclonal cells were selected and transferred into 24-well plates for scale-up culture. Then, the cells were divided into untreated cell group (control group), negative control group transfected with non-homologous vector (empty vector group), positive group transfected with the target sequence CSN5 shRNA (silencing group 1) and positive group 2 (silencing group 2).

Cell invasion assay

Chemoattractant FN was smeared onto the membrane outer surface of the Transwell chamber coated with Matrigel; 5 × 10⁵ tumor cells were added to the upper chamber while 600 µl DMEM containing 10% fetal bovine

serum was added to the lower chamber. After culturing for 24 h, the cells were fixed and stained to observe tumor cell infiltration across the membrane in order to determine the changes in the in vitro invasibility of tumor cells.

Cell adhesion assay

The 96-well plates coated with Matrigel were blocked with 3% BSA for 30min and washed 3 times with PBS. Then, 5 × 10⁴ tumor cells were added and cultured for 2 h. After washing 5 times with PBS, MTS reagent was added to continue the culture for 2 h. The absorbance was measured with a microplate reader to observe the changes in the in vitro adhesibility of tumor cells.

Determination of cell cycle changes using flow cytometry

In 6-well plates, 3 × 10⁵ cells were added and cultured for 24 h. After digestion and centrifugation, the cells were fixed with 500 µl of pre-cooling 70% ethanol at 4°C overnight. Then, 300 µl of RnaseA-containing PI staining solution was added to incubate at room temperature for 30 min. After washing with PBS, the absorbance was measured at 488nm with flow cytometry.

Western blot assay

In 6-well plates, 3 × 10⁵ cells were cultured for 24 h. After digestion and centrifugation, total cell proteins were extracted from the cells using RIPA lysis method; the protein concentration was determined with BCA method. Then, 100 µg proteins were applied onto 12% SDS-PAGE for electrophoresis and then transferred onto nitrocellulose membrane. The membrane was blocked in 5% skim milk at 4°C overnight. The primary antibodies (CSN5, 1:500; CD44, 1:500; MMP-2, 1:500; MMP-9, 1:500; CDK1, 1:500; P53, 1:300; P27, 1:300; AKT, 1:1000; p38, 1:1000; p-p38, 1:1000 α -actin, 1:300; p-AKT, 1:200; β -actin, 1:5000) were added to incubate at 4°C overnight. After washing 3 times, the secondary antibody was added to incubate at room temperature for 1h prior to ECL fluorescence imaging.

Real-time PCR assay

In 6-well plates, 3 × 10⁵ cells were cultured for 24h. After digestion and centrifugation, the

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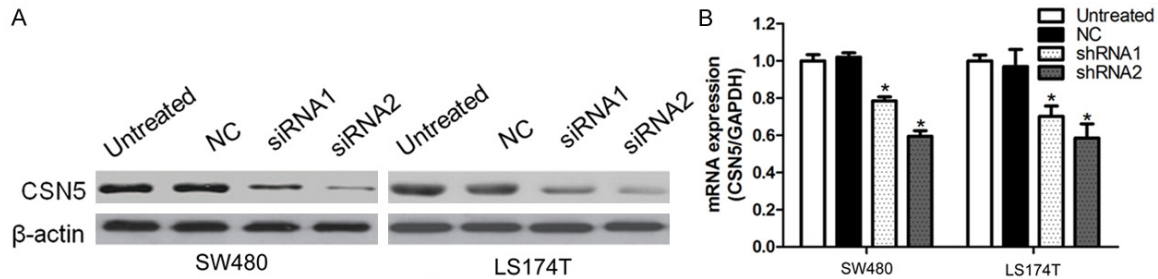


Figure 1. CSN5 shRNA inhibited CSN5 protein and mRNA expression. SW480 and LS174T cell lines were transfected with CSN5 shRNA1 and shRNA2 or NC, then CSN5 protein and mRNA expression were measured by western blot and real-time PCR. The statistical significance was considered as * $P < 0.05$ where compared with untreated group.

total RNA was extracted using Trizol method. SYBR Green I-labeled PCR product was used for fluorescent quantitative PCR reaction to obtain the respective CT values. The $2^{-\Delta\Delta CT}$ method was employed to analyze the differences of relative gene expression in each sample using GAPDH as the internal reference gene. The gene primer sequence was given in **Table 1**.

Determination of cytokine level using liquid chips

In 6-well plates, 3×10^5 cells were cultured for 24 h. Then, 100 μ l supernatant was obtained. Cytokines TGF- β , IL-1 β and IL-6 were determined using Luminex 2000 liquid chip analysis system in accordance with kit instructions.

Statistical analysis

SPSS10.0 statistical software was used for data analysis. The relevant data were expressed as mean \pm standard deviation ($\bar{x} \pm s$). One-way ANOVA was carried out. When $P < 0.05$, the difference had statistical significance.

Results

Identification of CSN5 silencing SW480 and LS174T cell lines

Western Blot and Real-time PCR results showed that CSN5 protein and mRNA expression was significantly down-regulated in CSN5 silencing group 1 and CSN5 silencing group 2 both in SW480 and LS174T cell lines (**Figure 1**); CSN5 protein expression was significantly lower in CSN5 silencing group 2 than in CSN5 silencing group 1.

Effects of CSN5 silencing on tumor cell adhesion, invasion and cell cycle

The cell adhesion assay result showed that the cell adhesibility was significantly lower in CSN5 silencing group than in control group and empty vector group. The cell adhesion rate in CSN5 silencing group 1 and CSN5 silencing group 2 was 43.7% and 28.5% of the control group, respectively ($P < 0.05$). Transwell results showed that the cell in vitro invasibility was significantly lower in CSN5 silencing group than in control group and empty vector group. The flow cytometric results showed that the cell cycle was arrested in G0/G1 phase after CSN5 expression was down-regulated (**Figure 2**).

Effects of silencing CSN5 expression on tumor cell-related gene and signal transduction molecule expression

Western Blot and Real-time PCR results showed that, in CSN5 silencing group 1 and CSN5 silencing group 2, the expression of CD44, MMP-2/9, CDK1, α -actin and protein and mRNA was significantly down-regulated, also p-AKT and p-p38, while the expression of P27 and P53 protein and mRNA was significantly up-regulated. Reporter gene test results showed that, in CSN5 silencing group 1 and CSN5 silencing group 2, NF- κ B and Twist transcriptional activity significantly decreased (**Figure 3**).

Effects of silencing CSN5 expression on cytokine expression in tumor cells

The liquid chip results showed that, in CSN5 silencing group 1 and CSN5 silencing group 2, TGF- β , IL-1 β and IL-6 secretion significantly decreased ($P < 0.05$) (**Figure 4**).

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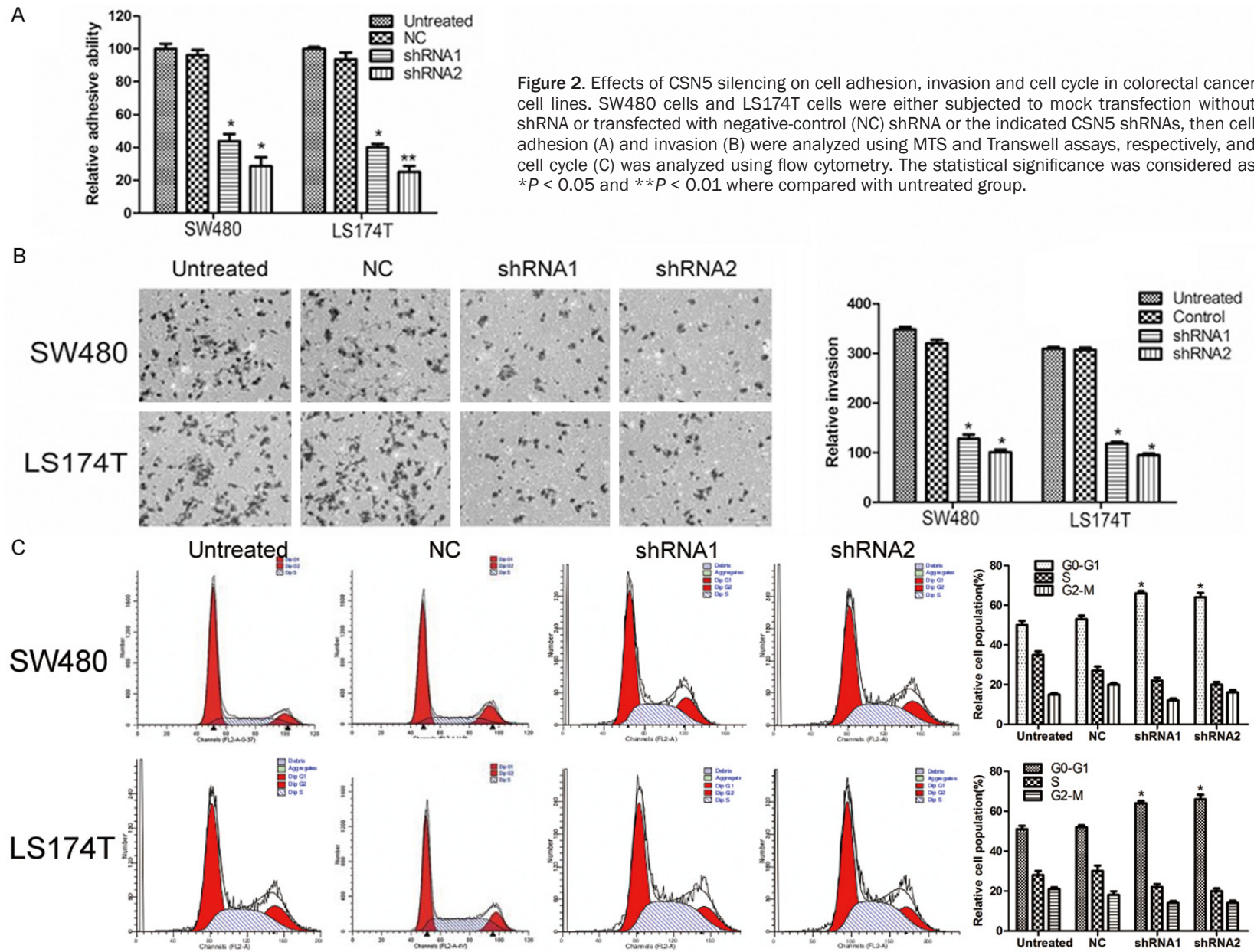


Figure 2. Effects of CSN5 silencing on cell adhesion, invasion and cell cycle in colorectal cancer cell lines. SW480 cells and LS174T cells were either subjected to mock transfection without shRNA or transfected with negative-control (NC) shRNA or the indicated CSN5 shRNAs, then cell adhesion (A) and invasion (B) were analyzed using MTS and Transwell assays, respectively, and cell cycle (C) was analyzed using flow cytometry. The statistical significance was considered as * $P < 0.05$ and ** $P < 0.01$ where compared with untreated group.

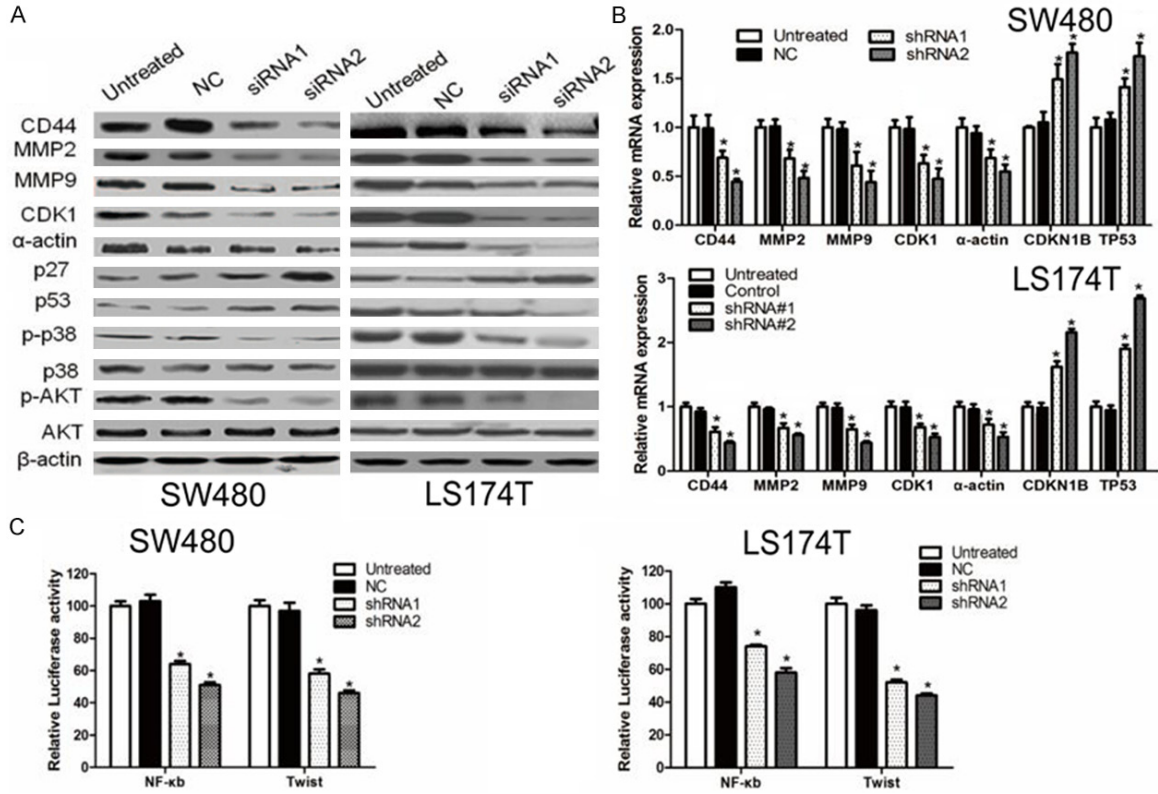


Figure 3. The effect of CSN5 silencing on expression of tumor related genes in colorectal cancer cell lines. A. SW480 cells and LS174T cells were either subjected to mock transfection without shRNA (lane 1) or transfected with negative-control (NC) shRNA (lane 2) or the indicated CSN5 shRNAs (lanes 3 and 4). A. Immunoblotting for the proteins associated with invasion and cell cycle. Cell lysates were prepared and subjected to ECL-western blotting by β-actin was as the internal control. Results are representative of three independent experiments. B. Real-time PCR was conducted to determine the mRNA levels of above mentioned genes. C. The transcriptional activity of NF-kb and Twist in cells by reporter gene assay. The statistical significance was considered as $**P < 0.01$ where compared with control.

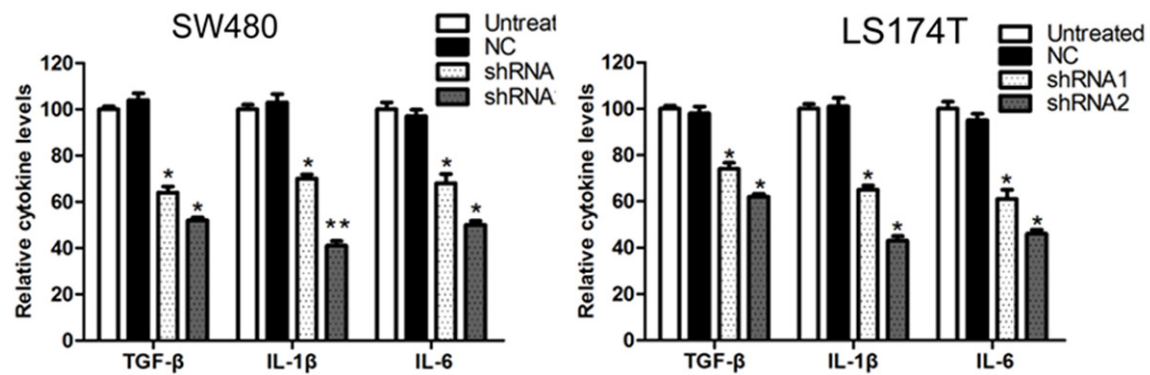


Figure 4. The effect of CSN5 silencing on several cytokines expression in colorectal cancer cell lines. SW480 cells and LS174T cells were either subjected to mock transfection without shRNA or transfected with negative-control (NC) shRNA or the indicated CSN5 shRNAs, then cytokine TGF-β, IL-1β and IL-6 levels were detected by liquid chip method. The statistical significance was considered as $*P < 0.05$ where compared with control.

Discussion

Tumor invasion is a complex process in which a variety of factors interact with each other.

CSN5, over-expressed in colorectal cancer and other human tumors, interacts with adhesion molecules, matrix metalloproteinases and a variety of other proteins to regulate tumor

cell growth and invasion [6, 8, 12]. This study used RNAi technology to silence CSN5 expression in human colorectal cancer SW480 and LS174T cells. It was observed that CSN5 silencing significantly inhibited the in vitro invasibility and adhesibility of tumor cells. In the meantime, it was found that CSN5 silencing significantly inhibited CD44 and MMP-2/9 expression, which may be one of the mechanisms for CSN5 to regulate tumor invasibility and adhesibility.

In this study, silencing CSN5 expression in SW480 and LS174T cells not only arrested the cell cycle in G0/G1 phase but also significantly promoted cell cycle-related genes P53 and P27 expression, and down-regulated CDK1 expression, suggesting that CSN5 plays an important role in tumor cell cycle regulation and plays a certain role in the regulation of P53 and other cell cycle-related gene expression.

TGF- β , IL-1 β , IL-6 and other cytokines play an important role in tumor invasion process [2, 3, 11, 15]. These cytokines not only promote tumor cell proliferation and invasion but also are involved in regulating tumor signal transduction. TGF- β can up-regulate oncogene expression and activate AKT pathway [14, 18, 19]. IL-6 and IL-1 β can exert tumor-promoting activity through cascade reaction [4, 5]. NF- κ B can participate in the inflammation-induced tumor invasion process to induce and enhance the activity of multiple transcription factors (e.g. Twist) responsible for tumor invasion. AKT, as one of the important downstream genes of CSN5 gene, plays a key role in tumor-related signaling pathways to regulate multiple tumor-related gene expression and transcription factor activity [7, 9]. In this study, silencing CSN5 expression down-regulated NF- κ B and Twist transcriptional activity, inhibited TGF- β , IL-1 β and IL-6 secretion, and regulated tumor-related gene expression, which may be related to the inhibition of AKT phosphorylation.

Disclosure of conflict of interest

None.

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