Pseudogene CSPG4P12 inhibits colorectal cancer progression by attenuating epithelial-mesenchymal transition

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Abstract

Colorectal cancer is one of the most common malignant cancers. Pseudogenes have been identified as oncogenes or tumor suppressor genes in the development of various cancers. However, the function of pseudogene CSPG4P12 in colorectal cancer remains unclear. Therefore, the aim of this study was to investigate the potential role of CSPG4P12 in colorectal cancer and explore the possible underlying mechanism. The difference of CSPG4P12 expression between colorectal cancer tissues and adjacent normal tissues was analyzed using the online Gene Expression Profiling Interactive Analysis 2 (GEPIA2) database. Cell viability and colony formation assays were conducted to evaluate cell viability. Transwell and wound healing assays were performed to assess cell migration and invasion capacities. Western blot was used to measure the expression levels of epithelial-mesenchymal transition-related proteins. Colorectal cancer tissues had lower CSPG4P12 expression than adjacent normal tissues. The overexpression of CSPG4P12 inhibited cell proliferation, invasion, and migration in colorectal cancer cells. Overexpressed CSPG4P12 promoted the expression of E-cadherin, whereas it inhibited the expression of vimentin, N-cadherin, and MMP9. These findings suggested that CSPG4P12 inhibits colorectal cancer development and may serve as a new potential target for colorectal cancer.

Key words: Colorectal cancer; Pseudogene; CSPG4P12; EMT; Mechanism

Introduction

Colorectal cancer (CRC) is the third most common cancer and the second leading cause of cancer-related deaths worldwide with an estimated number of 1.88 million new cases and about 915,880 deaths worldwide in 2020 (1). The initiation, progression, and metastasis of CRC is a multi-step process caused by the accumulation of genetic and epigenetic alterations (2). In recent years, studies revealed that around 16–17% CRC patients carried pathogenic germline variants (3,4). Epigenetic modifications, including DNA methylation, histone modifications, long noncoding RNAs (lncRNAs) and microRNAs (miRNAs), play an important role in the development of CRC (2). During the last decade, due to earlier diagnosis through screening, advances in surgical techniques, and novel targeted therapies, the 5-year relative survival rate for CRC has greatly improved from 50 to 65% (5). However, stage IV CRC patients still have a poor prognosis with 11–15% 5-year survival rate (5). Therefore, there is an urgent need to explore new therapeutic targets of CRC.

Pseudogenes usually refer to a DNA segment structurally similar to a gene but lacking coding function. Recently, increasing evidence indicates that pseudogenes are involved in gene regulation and play important roles in the process of tumor progression (6–8). For example, RP9P promotes colorectal cancer progression by regulating the miR-133a-3p/FOXQ1 axis (9). FLT1P1 inhibits both VEGFR1 and non-cognate VEGF-A expression, suppressing tumor cell proliferation and xenograft tumor growth (10).

Pseudogenes share high sequence homology with their parental genes (11). Pseudogenes and coding genes
can talk with each other by competing for the same microRNAs, acting as competing endogenous RNAs (ceRNAs) (11). For example, PTENP1 regulates its parent gene PTEN through the ceRNA mechanism (8). Chondroitin sulfate proteoglycan 4 (encoded by CSPG4), a cell surface proteoglycan, plays multiple roles in tumor growth and metastasis (12,13). CSPG4P12, as a pseudogene of CSPG4, has been reported to inhibit non-small cell lung cancer (NSCLC) development and tumorigenesis by activating the p53/Bcl2/Bax mitochondrial apoptotic pathway in our previous study (14).

In the present study, we evaluated whether pseudogene CSPG4P12 regulated the proliferation and metastasis capability of CRC and sought to further explore its potential molecular mechanisms. Our findings may provide a novel insight into the treatment of CRC.

**Material and Methods**

**Differential expression analysis**

The Gene Expression Profiling Interactive Analysis 2 (GEPIA2) database (http://gepia2.cancer-pku.cn/#analysis), a newly developed interactive web server for analyzing RNA sequencing expression data, was used to evaluate the differential expression of CSPG4P12 between CRC cancer tissues and adjacent normal tissues. A \(\log_2\) fold change (FC) > 1 and \(P\)-value < 0.05 were set as the cut-off criteria. After entering the gene symbols into the “Gene” column, the expression box plots were automatically generated on the webpage.

**Cell culture and plasmid transfection**

Human colorectal cancer cell lines (LOVO, Caco-2, and HCT116) and the human normal colonic epithelial cell line (NCM460) were obtained from American Type Culture Collection (ATCC) (USA). Cells were maintained in RPMI-1640 (EallBio Life Sciences, China) supplemented with 10% fetal bovine serum (FBS) (EallBio Life Sciences) and 1% penicillin and streptomycin (P/S) (EallBio Life Sciences) at 37°C in an incubator with 5% CO\(_2\).

Cells were seeded onto six-well plates with a density of \(1 \times 10^5\) cells/well and transfected at ~80% confluence with 1 \(\mu\)g CSPG4P12-pUC57 or control plasmid pUC57 using Neofect DNA transfection reagent (Neofect Biotech Co., Ltd., China) according to the manufacturer’s instructions. After 24 h, the transfected cells were collected for subsequent experiments. The CSPG4P12-pUC57 plasmid was constructed by Changzhou RuiBo Bio-Technology Co. (China) and the resulting constructs were verified by Sanger sequencing (14). Experiments were repeated three times for each.

**Reverse transcription-quantitative PCR (RT-qPCR)**

Total RNA was extracted from CRC cells using TRizol \(\text{\textregistered}\) reagent (Beijing Jinbaite Biotechnology Co., Ltd., China). Reverse transcription was carried out with 2 \(\mu\)g RNA, which served as a template to synthesize cDNA, according to the manual of the reverse transcription kit (Zhongshi Gene Technology Co., Ltd., China). cDNA was used for detection of CSPG4P12 mRNA using SYBR Green qPCR Mix (Zhongshi Gene Technology Co., Ltd.). GAPDH was used as an internal control gene. Primer sequences were reported in our previous study (14). The qPCR condition was 95°C for 5 min, followed by 40 cycles at 95°C for 10 s and 60°C for 20 s. The change of expression unit was calculated using the formula \(2^{-\Delta\Delta CT}\). Experiments were repeated three times.

**Bioinformatics analysis**

This analysis used combined gene expression data from the TCGA COAD cohort (https://portal.gdc.cancer.gov/projects/TCGA-COAD) and READ cohort (https://portal.gdc.cancer.gov/projects/TCGA-READ), which contained gene expression data from 638 tumor tissues. All data were downloaded from TCGA website and analyzed using R (version: 4.2.2) to obtain CSPG4P12 co-expressed genes. Sangerbox has a user-friendly interface and supports differential analysis, correlation analyses, pathway enrichment analysis, weighted correlation network analysis, and others (15). We uploaded the CSPG4P12 co-expressed gene data to the Sangerbox website and used the Sangerbox website for Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis. We used the GEPIA2 database (http://gepia2.cancer-pku.cn/#index) to analyze the correlation between CSPG4P12 and common mutated genes in colorectal cancer cells.

**Cell counting kit-8 (CCK8) assay**

Cell viability was evaluated by CCK8 assay. The transfected CRC cells (LOVO and Caco-2) were seeded at a density of \(2 \times 10^3\) cells/well in 96-well plates and incubated for 0, 24, 48, and 72 h. Subsequently, 10 \(\mu\)L CCK8 (Mei5 Biotechnology Co., Ltd., China) was added to each well and incubated for another hour at 37°C. Absorbance values at 450 nM were measured using a microplate reader (Thermo scientific, USA). Experiments were repeated three times.

**Colony formation assay**

The transfected CRC cells were harvested and reseeded onto 6-well plates at a density of \(2 \times 10^3\) cells/well. After incubation for 14 days, cells were then fixed with methanol and stained with 1% crystal violet dye (0.1% w/v). Colonies were photographed (Canon, Japan) and counted by ImageJ software (NIH, USA). Experiments were repeated three times.

**Wound-healing assay**

Transfected and non-transfected CRC cells (LOVO and Caco-2) were seeded onto six-well culture plates containing 2 mL RPMI 1640 medium and 10% FBS at a
density of $2 \times 10^3$ cells/well until they reached 80% confluency. The cell monolayer was scratched with a 200-μL pipette tip. The cells were then cultured in RPMI 1640 medium for 48 h. Cells and scratch closure were observed under an inverted light microscope (Nikon, Japan) and images were captured at 0, 24, and 48 h after scratching and the cell-free area was measured using ImageJ software (NIH). Experiments were repeated three times.

Transwell assays
Transwell assays were performed to assess cell migration and invasion. For these assays, 5–200×10^3 transfected cells in 200 μL RPMI 1640 medium were seeded onto the 8-μm pore size upper chamber (JET BIOFIL, China) with Matrigel (Corning, USA) (invasion) or without Matrigel (migration). Matrigel matrix (8–11 mg/mL) was mixed with RPMI 1640 medium at 1:4 and the final concentration of Matrigel used in transwell assays was 1.6–2.2 mg/mL. The lower chamber was supplemented with 600 μL RPMI 1640 medium containing 20% FBS. After incubation at 37°C for 24 or 48 h, cells that passed through the filter were fixed with methanol and stained with 0.1% crystal violet solution. After imaged under an inverted light microscope, cells were measured using the ImageJ software. Experiments were repeated three times.

Western blot and antibodies
Total proteins were extracted using RIPA buffer (Beijing Zoman Biotechnology Co., Ltd, China) supplemented with the protease inhibitor cocktail (Beijing Zoman Biotechnology Co., Ltd) according to the manufacturer’s instructions. Proteins (20 μg) were separated by 10% SDS-PAGE (Biotides, China) and then transferred to polyvinylidene difluoride (PVDf) membranes (Millipore, USA). The membranes were blocked in 5% milk for two hours and incubated with primary antibodies at 4°C overnight. Subsequently, the membranes were incubated with secondary antibodies at 37°C for one hour. Finally, proteins were visualized by using enzyme-linked chemiluminescence detection kit (ECL) and images were captured by a chemiluminescence imaging system (Azure Biosystems C300). The pixels from the western blot membranes were quantified by ImageJ software (NIH). Experiments were repeated three times.

NCM Universal Antibody Diluent was purchased from New Cell Molecular Biotech Co., Ltd (China). Anti-mouse IgG antibody (H+L) (1:10000) and anti-rabbit IgG (H+L) antibody (1:10000) were purchased from Seracare (USA). Anti-beta actin (1:10000), anti-N-cadherin (1:1000), and anti-E-cadherin (1:1000) antibodies were purchased from GeneTex (USA). Anti-MMP-9 (1:1000) and anti-vimentin (1:5000) antibodies were purchased from Proteintech (China).

Statistical analysis
IBM SPSS Statistics 26.0 software (USA) was used to perform statistical analyses. GraphPad Prism 9.0 software (USA) was used to draw graphs. A paired t-test was used to analyze the difference in CSPG4P12 expression between CRC cancer tissues and adjacent normal tissues. One-way ANOVA followed by Bonferroni’s post hoc correction was utilized to evaluate the result of wound healing assay, transwell assay, and western blot. P < 0.05 was considered statistically significant.

Result
CSPG4P12 expression was decreased in CRC cells and cancer tissues
RT-qPCR revealed significantly lower levels of CSPG4P12 expression in CRC cells. The expression level of CSPG4P12 in Caco-2, LOVO, and HCT116 cells were 0.15-, 0.34-, and 0.36-fold compared with NCM460 cells (P < 0.05; Figure 1A). The differential expression of CSPG4P12 in CRC and normal colon-rectal tissues was analyzed by the RNA-seq data from TCGA database and GTEx database. A total of 275 colon adenocarcinoma (COAD) and 349 healthy tissues from TCGA, and 92 rectum adenocarcinoma (READ) tissues and 318 healthy tissues from GTEx were used (Figure 1B). We found that the expression of CSPG4P12 in CRC tissues was significantly lower than that in the adjacent normal tissues (P < 0.05; Figure 1B). KEGG enrichment analysis of CSPG4P12 co-expressed genes showed that they were mainly enriched in cancer-related pathways such as G2M, MYC, and E2F (Figure 1C). Our analysis also showed no correlation between CSPG4P12 and expression levels of commonly mutated genes in CRC patients (P53, KRAS, c-myc, Hras, Nras, Myb) (Supplementary Figure S1).

Overexpression of CSPG4P12 inhibited the growth of CRC cells
CSPG4P12-pUC57 overexpressed plasmid was transfected into CRC cells (LOVO and Caco-2). After 24 h, the expression levels of CSPG4P12 in LOVO and Caco-2 cells were elevated by 24.7- and 280.2-fold, respectively (Figure 2). The CCK-8 assay results showed that the number of CRC cells with overexpression of CSPG4P12-pUC57 (P < 0.01) (Figure 3A and B). The colony formation experiment also showed that the number of CRC cell colonies in the CSPG4P12-pUC57 group was significantly lower than that in the control group (Figure 3C–F). These results demonstrated that CSPG4P12 may promote the proliferation of CRC cells.

Overexpression of CSPG4P12 decreased cell migration and invasion ability
The CRC cells with overexpression of CSPG4P12 showed a decreased migratory ability to close the wound
at 24 and 48 h (Figure 4A–D). In line with this finding, our data from transwell migration analysis demonstrated that upregulation of CSPG4P12 repressed migration of LOVO and Caco-2 cells (Figure 4E and F). Furthermore, we observed that CSPG4P12 overexpression suppressed cell invasive ability in LOVO and Caco-2 cells (Figure 4G and H). Our results indicated that CSPG4P12 regulated cell migration and invasion in CRC cancer.

Overexpression of CSPG4P12 inhibited EMT

Epithelial-mesenchymal transformation (EMT) is the first step of metastasis and plays an essential role in CRC progression. Therefore, we measured the expression of EMT markers, including E-cadherin, N-cadherin, vimentin, and matrix metalloproteinase-9 (MMP9) in CRC cells with CSPG4P12 overexpression. Western Blot assay showed that the overexpression of CSPG4P12 inhibited the expression of vimentin, N-cadherin, and MMP9, but promoted the expression of E-cadherin, which suggested an elevated EMT progression (Figure 5A–C). These results demonstrated that overexpression of CSPG4P12 could promote the progression of EMT in CRC.

Discussion

Tumor metastasis is still a challenge affecting the prognosis of CRC patients. Approximately 33% of patients with CRC will develop metastases (16). Growing evidence indicates that pseudogenes, a type of long noncoding RNA (lncRNA), regulates the progression of CRC by promoting proliferation, invasion, and migration (7). For example, a study revealed that DUXAP8 targeted miR-577 and promoted the expression of oncogene RAB14, which promoted colon cancer cell proliferation and progression (17).
The decreased expression of CSPG4P12 in CRC could be attributed to several molecular mechanisms that regulate gene expression in cancer. Our analysis revealed that CSPG4P12 co-expressed genes are significantly enriched in the E2F and MYC pathways, which are pivotal in CRC development. The E2F and MYC pathways are known to be tightly regulated under normal physiological conditions but are frequently overactivated or disrupted in cancerous cells (18,19). The enrichment of CSPG4P12 co-expressed genes in these pathways suggested that CSPG4P12 may act as a modulator of these critical oncogenic pathways. The down-regulation of CSPG4P12 in CRC could, therefore, be a consequence of the cancer cells’ attempt to escape the normal regulatory mechanisms that limit cell proliferation and promote differentiation. By decreasing CSPG4P12 expression, CRC cells may enhance the activity of the E2F and MYC pathways, promoting uncontrolled cell division and progression of the cancer.

CSPG4, which is the parental gene of CSPG4P12 (12), is involved in tumor carcinogenesis, particularly in cancer proliferation, motility, and metastatic spread, and has been used as an independent biomarker for cancer prognosis (20). Our study disclosed that CSPG4P12 acted as a crucial regulator in CRC development, and overexpression of CSPG4P12 restrained CRC cell progression and metastasis. Consistent with this finding, our previous study (14) revealed that expression of CSPG4P12 was decreased in NSCLC tissues compared to normal lung tissues, and overexpressed CSPG4P12 significantly inhibited lung cancer cell proliferation, migration, invasion, and adhesion. CSPG targeting antibody-drug conjugate showed a strong toxic effect on cancer cells in a concentration-dependent manner (21). Furthermore, CSPG4-specific mAb has been shown to inhibit growth, adhesion, and migration of cancer cells in vitro, and significantly reduces the tumorigenic power of cancer cells and mitigates metastases and recurrence in vivo (22). CSPG4 is also thought to play important roles in the progress of wound healing (23). In present work, the wound-healing assay and transwell experiment validated...
the stimulatory effect of CSPG4P12 on cell migration and invasion.

EMT plays critical roles in cancer metastasis, resulting in poor prognosis in CRC patients (24). In the present study, we showed that CSPG4P12 overexpression mediated the inhibition of EMT. Previous studies revealed that knockdown of CSPG4 can reduce expression of EMT markers (E-cadherin, N-cadherin) and key EMT regulator (Snail), and then inhibit tumor migration/invasion (25,26). In our study, western blot experiments revealed that epithelial markers (E-cadherin) were significantly increased, while mesenchymal markers (vimentin, N-cadherin) were decreased by CSPG4P12. A growing body of clinical and experimental studies have shown the prognostic value of these EMT-related proteins in CRC patients. For example, studies have reported that aberrant regulation of EMT-related epithelial (E-cadherin) and mesenchymal (vimentin, N-cadherin) markers have been identified in CRC and are associated with increased rate of cancer recurrence, metastasis, and poor prognosis of CRC patients (27–30). E-cadherin is a key component of the adhesion junctions and regulates CRC proliferation.

**Figure 4.** Effects of CSPG4P12 overexpression on migratory and invasive abilities of colorectal cancer cells. Detection and quantification of cell migration using wound healing assay (magnification, × 40, scale bar 250 μM) (A–D). Detection of cell migration and invasion using Transwell assay (magnification, × 100, scale bar 100 μM) (E and G), the results of which were quantified (F and H). Data are reported as means and SD. **P < 0.01, ***P < 0.001, and ****P < 0.0001 (Student’s t-test). CSPG4P12: chondroitin sulfate proteoglycan 4 pseudogene 12. Experiments were done in triplicate.
and migration (31). Kuphal and Bosserhoff (32) reported that N-cadherin is directly regulated by E-cadherin, and loss of E-cadherin induces N-cadherin expression in tumorigenic EMT.

MMP9 contributes to various phases of colorectal cancer progression, including invasion, EMT, and angiogenesis (33,34). We found that overexpression of CSPG4P12 could significantly decrease the expression of MMP9.

We acknowledge the importance of in vivo experiments or 3D co-culture models in bridging the gap between in vitro results and clinical applicability. Although resource constraints precluded us from conducting these studies at this stage, we emphasize the value such experiments would bring to the field. Specifically, future research employing in vivo and 3D co-culture models could further validate CSPG4P12 as a key player in CRC progression and provide deeper insights into its mechanisms of action. These studies would not only indicate the therapeutic potential of targeting CSPG4P12 but also facilitate the development of more effective and personalized treatment strategies for CRC patients.

In conclusion, our current study lays the groundwork for understanding CSPG4P12’s role in CRC. We hope that our work will inspire and pave the way for these essential investigations, moving us closer to novel interventions for CRC.

Figure 5. Effects of CSPG4P12 overexpression on epithelial-mesenchymal transition marker protein expression. A, Detection of protein expression using western blotting, which was semi-quantified (B and C). Data are reported as means and SD. *P < 0.05, **P < 0.01, and ****P < 0.001 compared to its own control (Student’s t-test). CSPG4P12, chondroitin sulfate proteoglycan 4 pseudogene 12. Experiments were done in triplicate.

References


