

Long non-coding RNA SSC4D as a potential diagnostic biomarker promotes gastric cell proliferation and metastasis

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ABSTRACT

Background: Scavenger receptor cysteine-rich family member with 4 domains (SSC4D) is aberrantly expressed in gastric cancer (GC) based on bioinformatics analysis. However, its function has not been studied in GC. **Materials and Methods:** The relation of SSC4D with GC patient survival and diagnosis was investigated based on bioinformatics analysis and clinical information from forty-seven patients. SSC4D was silenced by shRNAs and the impact of SSC4D silencing on GC cell proliferation, migration, and invasion was analyzed. Xenograft nude mice were also used to reveal the function of SSC4D on GC carcinogenesis at a pre-clinical level. **Results:** The knockdown of SSC4D inhibits GC cell malignancy as well as tumorigenesis. **Conclusion:** SSC4D exerts oncogenic effects on GC cell growth, migration, invasiveness and epithelial cell mesenchymal transition (EMT) and tumor growth.

Keywords: Gastric cancer, SSC4D, biomarker, migration, invasion.

INTRODUCTION

Gastric cancer (GC) is a common global disease, ranking the fifth in cancer incidence and the fourth in cancer mortality globally. An estimated over 1000,000 new GC cases are reported in 2020 across the world, occupying 5.6% of all cancer incidence ⁽¹⁾. GC with a crude incidence of 30.00/100,000 is the third most frequently diagnosed cancer in China, which poses a considerable burden to public health ⁽²⁾. The prospects for metastatic GC patients are inimical, and the median survival rate was no more than a year ⁽³⁾. Endoscopic resection is mainly recommended for the early GC treatment, and chemotherapy remains the cornerstone for metastatic GC therapy ⁽⁴⁾. Radiotherapy is also an important part in GC therapy to improve patient survival ^(5,6). However, the clinical outcome of the chemotherapy and radiation therapy was affected by drug resistance as well as unavoidable severe toxicity ⁽⁷⁾. Targeted therapy is also proposed with improved clinical outcomes for GC patients ⁽⁸⁾. It is of eminent significance to deepen the molecular understanding to improve the therapeutic intervention for GC.

Classification based on molecular biomarkers contributes to the improved precision oncology ^(9,10). Scavenger receptor cysteine rich family member with 4 domains (SRCRB4D), also known as SSC4D, with the location in the 7q11.23 region, is a member of the

the highly conserved scavenger receptor cysteine-rich (SRCR) superfamily proteins, which are regarded to function in epithelia and the immune system and related to the progression of various diseases, including cancer ⁽¹¹⁾. Knockdown of the SRCR proteins might increase the sensitivity of cells to irradiation, which might shed light on the cancer targeted therapy ⁽¹¹⁾. The high expression of SSC4D is thought to be implicated in gastric cancer progression ⁽¹²⁾. SSC4D has been found to be enriched in cervical carcinoma ⁽¹³⁾, ovarian cancer ⁽¹⁴⁾, while differently expressed in Desmoplastic Small Round-Cell Tumor ⁽¹⁵⁾. It also displayed a potential regulatory role on the oncogenic pathways ⁽¹⁶⁾. Whereas, the exact function of SSC4D in GC is unexplored.

Our work explored the function of SSC4D in GC based on bioinformatics approaches and loss-of-function experiments. We also investigated the role of SSC4D in GC diagnosis and prognosis. The oncogenic role of SSC4D in GC was first revealed, which might provide novel target for GC treatment.

MATERIALS AND METHODS

Bioinformatics analysis

SSC4D expression profile in 375 GC tissues and 32 normal samples was obtained from the starbase

database ⁽¹⁷⁾. The survival analysis for GC patients (SSC4D high-/low-) was generated from the Kaplan-Meier (KM) Plotter online tool in GC based on patient transcriptomic data ⁽¹⁸⁾. Top 20 similar genes for SSC4D in stomach adenocarcinoma tissues were obtained from the GEPIA database ⁽¹⁹⁾. Gene Ontology (GO) analyses were performed based on the KOBAS online tool ⁽²⁰⁾. The DAVID 6.82 tool was applied for Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses.

Tissue specimens

Totally 47 GC tumor specimens and adjacent non-tumor stomach mucosa specimens were obtained from 47 GC patients receiving radical gastrectomy at Haining Traditional Chinese Medicine Hospital. No patients were treated with radiotherapy or chemotherapy prior to surgery. All the patient participants signed the written informed consent. All specimens were collected with the approval of the ethics committee of our hospital.

Cell culture, RNA extraction, and RT-PCR

We followed the methods of Haiming Liu *et al.* ⁽²¹⁾. The human GC cell lines (AGS, MKN45) were provided by American Type Culture Collection (ATCC, USA). RT-PCR were performed as previously described ⁽²²⁾. Total RNA isolation was performed using TRIzol Reagent (Beyotime, China) and cDNA was produced using a RevertAid First Strand cDNA Synthesis Kit (Thermo fisher, USA). PCR was conducted using LightCycler FastStart DNA MasterPLUS SYBR Green I mix (Roche, Germany) on the Roche Lightcycler 480 Sequence Detector (Roche). Specific Primers for SSC4D were obtained from ELK Biotechnology (China). The sequences of primers are shown below: SSC4D forward, 5'-TCACTACGAGGATGTGGCTGTC-3', reverse, 5'-TTCCATTTCGGCAGTGTCTAGG-3'; GAPDH forward, 5'-TCTCTTAGGCGCATCTACTTA-3', reverse, 5'-AGGAGGATTCATTACAGGCTAC-3'.

Cell transfection

The short hairpin RNAs (shRNAs) for SSC4D (sh-SSC4D) were obtained from ELK Biotechnology (China). The shRNA sequences for SSC4D are: 1: 5'-TCGACTGTTGTGAGCCCAATT-3', 2: 5'-GGGAAGAG-3'. Cell transfection was performed using Lipofectamine 3000 reagent (Invitrogen, USA). The lentivirus expressing sh-SSC4D (LV-sh-SSC4D) was packaged by Genepharma (Shanghai, China). An empty lentivirus vector was used as a negative control (NC).

Cell proliferation assays

GC cell proliferative capacity was evaluated as previously reported ⁽²²⁾. A Cell Counting Kit-8 assay kit (Solarbio, Beijing, China) was applied following the producer's guide. For colony formation assays, cells were grown into six-well culture plates followed by incubation at 37°C for fourteen days. After fixation

using 4% formaldehyde (Sigma-Aldrich, USA), colonies were subject to staining using 0.1% crystal violet (Solarbio, China) and number of colonies was counted using a light microscope (Olympus, Japan).

Transwell assays

Transwell cell chambers (Corning, China) with an 8-mm pore membrane were applied. After cell starvation for twelve hours using a serum-free medium, cells (5×10^5) were grown in the top chambers of the for the migration assays. For cell invasiveness assessment, cells (1×10^5) were inoculated in top transwell chambers coated by Matrigel (Corning, China). After incubation for a day, cells migrated or invaded in the lower chambers were fastened and dyed using 5% crystal violet (Solarbio, and their number was calculated in five randomly chosen fields at magnification 100 $\times$.

Western blotting

Western blotting analysis was conducted following the methods of Haiming Liu *et al.* ⁽²¹⁾. After PBS washing three times, cells were lysed using RIPA lysis buffer (Beyotime, China). A BCA assay was carried out for determining the protein concentration. Protein samples were electrophoresed and subsequently electrotransferred to a polyvinylidene fluoride membrane (Merck Millipore, USA). Subsequently, 5% nonfat milk was applied for blocking the membrane for one hour at ambient temperature. Next, each membrane section was incubated with the designated primary monoclonal antibody and incubated overnight at 4°C. The next day, all membranes were washed thrice using Tris Buffered Saline with Tween® 20 and then maintained with the secondary antibody (ab205718, 1:2000) for one hour at ambient temperature. The protein expression was analyzed using an (Bio-Rad, USA) imaging system. The relative protein expression was evaluated corresponding to the value of GAPDH. Primary antibodies were provided by Abcam (Shanghai, China): anti-E-cadherin (ab256580; 1:1000), anti-Fibronectin (ab268021; 1:1000), anti-Snail1 (ab216347; 1:1000), with GAPDH (ab8245; 1:1000) as the loading control.

In vivo analysis

Twenty BALB/c nude mice (male, 4-5 weeks, Vital River, Beijing, China) were injected subcutaneously with LV-NC and LV-sh-SSC4D-transfected MNK45 cells into their side of the axillary region. The animals were housed under pathogen-free conditions. The xenograft model was established by injecting infected MNK45 cells (1×10^7) to the back right side of nude mice. Body weight and tumor size of animals were recorded weekly. After four weeks, all animals were euthanized and the tumor samples were dissected for following analysis. Our work was strictly conducted following the ethical standards of Haining Traditional Chinese Medicine Hospital. The tumor volume

calculation was based on the formula: tumor volume (mm^3) = [length (mm) $\times$ width² (mm) $\times$ π]/6.

Statistical analysis

All assays were run repetitively three times. Results were exhibited as the mean value $\pm$ standard deviation and statistical comparison between two groups or among multiple groups was subject to t-test or analysis of variance (ANOVA) on the Statistical Product and Service Solutions statistical software (SPSS, USA). P values less than 0.05 indicates significance. The overall survival curve was generated using Kaplan Meier (KM) method. The GraphPad Prism software (GraphPad Software Inc., USA) was used for visualization of the results.

RESULTS

SSC4D Is a Potential Biomarker for GC

Based on starBase v3.0, SSC4D is significantly upregulated in stomach adenocarcinoma tissues

(figure 1A). According to KM plotter analysis, high SSC4D expression is associated with the lower rates of overall survival (figure 1B), progression-free survival (figure 1C) and post progression survival of GC patients (figure 1D). We also collected tumor samples and paired nontumor samples from GC patients in our hospital and found that SSC4D is significantly upregulated in these GC tissues (figure 1E). The overall survival of SSC4D high-/low- GC patients was also analyzed, and we identified that high SSC4D expression predicted poor prognosis of GC patients (figure 1F). Also, based on the receiver operating characteristic (ROC) curve, the ability of SSC4D expression in discriminating cancerous tissue from normal tissue. As shown in figure 1G, the expression of SSC4D was of diagnostic value to discriminate normal samples from cancerous samples (area under the curve (AUC) = 0.8685, 95% CI: 0.7946-0.9424, $p < 0.0001$). Overall, SSC4D is of diagnostic as well as prognostic value in GC.

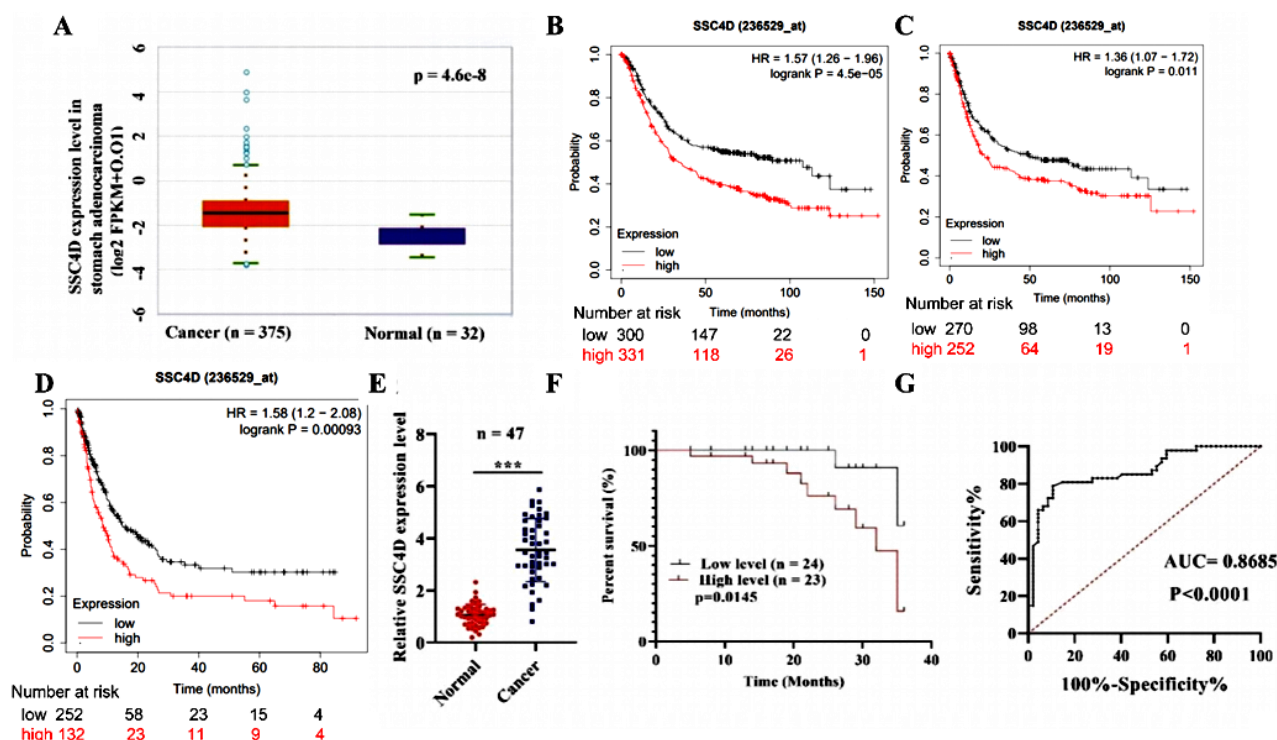


Figure 1. SSC4D is upregulated in GC with potential diagnostic and prognostic value.

(A) SSC4D level in GC was obtained from the starbase online tool. (B) The overall survival, (C) progression-free survival and (D) post progression survival in SSC4D-high/low- GC patients, which was based on Kaplan-Meier Plotter online tool. (E) SSC4D was upregulated in 47 GC cancer samples. (F) Overall survival curve of SSC4D high-/low- GC patients was plotted based on the KM method and log-rank test. (G) The ROC curve revealed the diagnostic value of SSC4D in GC. *** $p < 0.001$.

Relation between SSC4D expression and clinicopathological features in GC patients

We employed the χ^2 test to analyze the relation of SSC4D expression with different patient clinical characteristics (table 1). The χ^2 test results suggested that the SSC4D level was not evidently correlated with age, gender and grade of GC patients, but related to size of tumor, T staging, helicobacter pylori infection, as well as smoking ($p < 0.05$).

Table 1. Relation between SSC4D and clinical characteristics in GC patients.

Variables	Cases	Relative SSC4D expression		P values
		High (n=23)	Low (n=24)	
Age (Years)				
≤60	15	9	6	0.2989
>60	32	14	18	
Gender				
Female	32	15	17	0.6797
Male	15	8	7	
Size of tumor				
≤5 cm	21	5	16	*0.0020
>5 cm	26	18	8	
Grade				
I+II	20	8	12	0.2915
III+IV	27	15	12	
T Staging				
T1+T2	25	17	8	*0.0053
T3+T4	22	6	16	
Infection of Helicobacter pylori				
Yes	14	10	4	*0.0445
No	33	13	20	
Smoking				
Yes	26	17	9	*0.0121
No	21	6	15	

*P < 0.05 was regarded as the threshold value. χ^2 test was performed. GC:gastric cancer; T tumor.

Functional enrichment analysis of SSC4D and its top 20 similar genes

Top 20 similar genes to SSC4D in stomach adenocarcinoma were obtained from GEPIA and were listed in table 2. Functional enrichment analysis was performed on SSC4D and the 20 similar genes. KEGG analysis revealed that vitamin digestion and absorption and cholesterol metabolism had high enrichment ratio. These genes were also associated with the PPAR pathway (figure 2). GO analysis revealed the enrichment of these genes in some key terms including extracellular exosome, glycoprotein, glycation, and methylation (table 3).

Table 2. Top 20 similar genes for SSC4D in stomach adenocarcinoma.

Gene Symbol	Gene ID	PCC
AHSG	ENSG00000145192.12	0.89
TNP1	ENSG00000118245.2	0.88
TF	ENSG00000091513.14	0.86
APOA2	ENSG00000158874.11	0.85
SERPINC1	ENSG00000117601.13	0.82
CCL16	ENSG00000275152.4	0.81
SLC2A2	ENSG00000163581.13	0.81
AMBP	ENSG00000106927.11	0.8
HULC	ENSG00000276019.1	0.79
ACSM2B	ENSG00000066813.14	0.79
AFM	ENSG00000079557.4	0.78
HULC	ENSG00000251164.1	0.78
ALB	ENSG00000163631.16	0.78
AFP	ENSG00000081051.7	0.78
CPB2	ENSG00000080618.13	0.77
APOC1P1	ENSG00000214855.9	0.77
CPN2	ENSG00000178772.6	0.77
RP11-622A1.2	ENSG00000250436.1	0.77
APOA1	ENSG00000118137.9	0.77
CSN1S2AP	ENSG00000234124.6	0.77

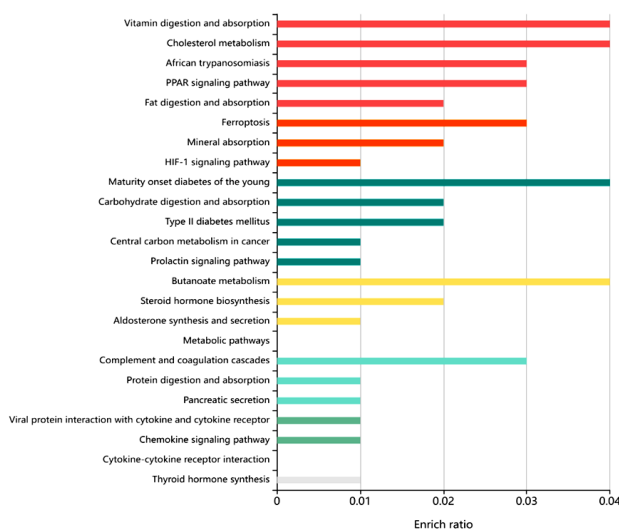
Data are derived from GEPIA database.

PCC: Pearson correlation coefficient; AHSG: Alpha-2 Heremans Schmid Glycoprotein; TNP1: transition nuclear protein 1; TF: transferrin; APOA2: apolipoprotein A2; SERPINC1: serpin family C member 1; CCL16: C-C motif chemokine ligand 16; SLC2A2: solute carrier family 2 member 2; AMBP: alpha-1-microglobulin/bikunin precursor; HULC: hepatocellular carcinoma up-regulated long non-coding RNA; ACSM2B: acyl-CoA synthetase medium chain family member 2B; AFM: afamin; ALB: albumin; AFP: alpha fetoprotein; CPB2: carboxypeptidase B2; APOC1P1: apolipoprotein C1 pseudogene 1; CPN2: carboxypeptidase N subunit 2; APOA1: apolipoprotein A1; CSN1S2AP: casein alpha s2 like A, pseudogene.

Table 3. Gene_Ontology analysis of SSC4D and its top 20 similar genes in stomach adenocarcinoma.

GOTERM-CC-DIRECT			
#	Category	Term	Kappa
1	GOTERM-CC-DIRECT	blood microparticle	1.00
2	GOTERM-CC-DIRECT	extracellular region	0.90
3	GOTERM-CC-DIRECT	extracellular exosome	0.79
4	UP-KEYWORDS	Glycoprotein	0.69
5	UP-KEYWORDS	Signal	0.59
6	UP-KEYWORDS	Secreted	0.59
7	UP-SEQ-FEATURE	signal peptide	0.59
GOTERM-BP-DIRECT			
#	Category	Term	Kappa
1	GOTERM-BP-DIRECT	platelet degranulation	1.00
2	GOTERM-CC-DIRECT	platelet alpha granule lumen	0.61
3	UP-KEYWORDS	Glycation	0.61
4	GOTERM-BP-DIRECT	retina homeostasis	0.61
5	GOTERM-CC-DIRECT	endocytic vesicle	0.61
6	GOTERM-MF-DIRECT	identical protein binding	0.61
7	GOTERM-CC-DIRECT	secretory granule lumen	0.61
8	UP-SEQ-FEATURE	glycosylation site:N-linked (Glc) (glycation)	0.61
9	UP-KEYWORDS	Methylation	0.61
10	GOTERM-CC-DIRECT	cytoplasmic vesicle	0.61
11	GOTERM-CC-DIRECT	nucleus	0.61
12	UP-KEYWORDS	Disease mutation	0.56

Similarity Score: Very High (0.75-1). GO: Gene Ontology, CC: Cell Component, BP: Biological Process, MF: Molecular Function.

**Figure 2.** KEGG enrichment analysis of SSC4D and its top 20 similar genes in stomach adenocarcinoma.

SSC4D knockdown inhibits GC cell proliferation

Two shRNAs specifically targeting SSC4D were designed to explore its biological function in GC cells. SSC4D was successfully silenced by shRNAs in AGS and MKN45 cells (figure 3A). Then, we delved into silencing SSC4D-caused impact on the proliferative ability of GC cells. As revealed by CCK-8 assays, silencing SSC4D hindered the GC cell growth (figure 3B). Moreover, the colony number of AGS and MKN45 cells exhibited significant decrease after silencing SSC4D (figure 3C). These findings indicate that SSC4D facilitates GC cell proliferation.

(A) SSC4D was silenced by shRNAs in GC cells according to RT-PCR (B) A CCK-8 assay evaluated GC cell proliferation under SSC4D knockdown. (C) Colony number of GC cells was analyzed in each transfection group. One way ANOVA analyzed the group difference, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

SSC4D knockdown reduces GC cell metastasis

The transwell assays were carried out to further explore the function of SSC4D on GC cells. Results elucidated that SSC4D silencing inhibited AGS and MKN45 cell migration as well as invasiveness (figure 4A and B). Epithelial cell mesenchymal transition (EMT) is reported to be associated with tumor metastasis, cancer metastasis can be affected via modulating EMT⁽²³⁻²⁵⁾. Thus, we further studied whether SSC4D affected EMT process in GC. The EMT

-related marker expression was measured and results confirmed that the knockdown of SSC4D downregulated E-cadherin while elevating fibronectin and SNA11 in GC cells (figure 4C and D). Overall, SSC4D enhances GC cell migration, invasion as well as the EMT process.

Transwell assays evaluated the effects of silencing of SSC4D on the (A) migration ability and (B) the invasiveness of GC cells. (C,D) The levels of EMT markers in GC cells was evaluated by western blotting and qRT-PCR. One way ANOVA was used to analyze the significant differences, ** $p < 0.01$, *** $p < 0.001$.

SSC4D knockdown suppresses tumor growth in vivo

MKN45 cells stably carrying low expression of SSC4D or control MKN45 cells were established after infection with lentivirus carrying sh-SSC4D and sh-NC. SSC4D expression was efficiently suppressed by LV-shRNAs (figure 5A). In the tumor-bearing mouse models, the tumor size was significantly reduced by SSC4D knockdown in comparison with the NC group (figure 5B). Moreover, mouse tumor weight as well as tumor volume was demonstrated to be decreased in the LV-sh-SSC4D group in comparison with NC group (figure 5C-D). Collectively, SSC4D knockdown suppresses mouse tumorigenesis *in vivo*.

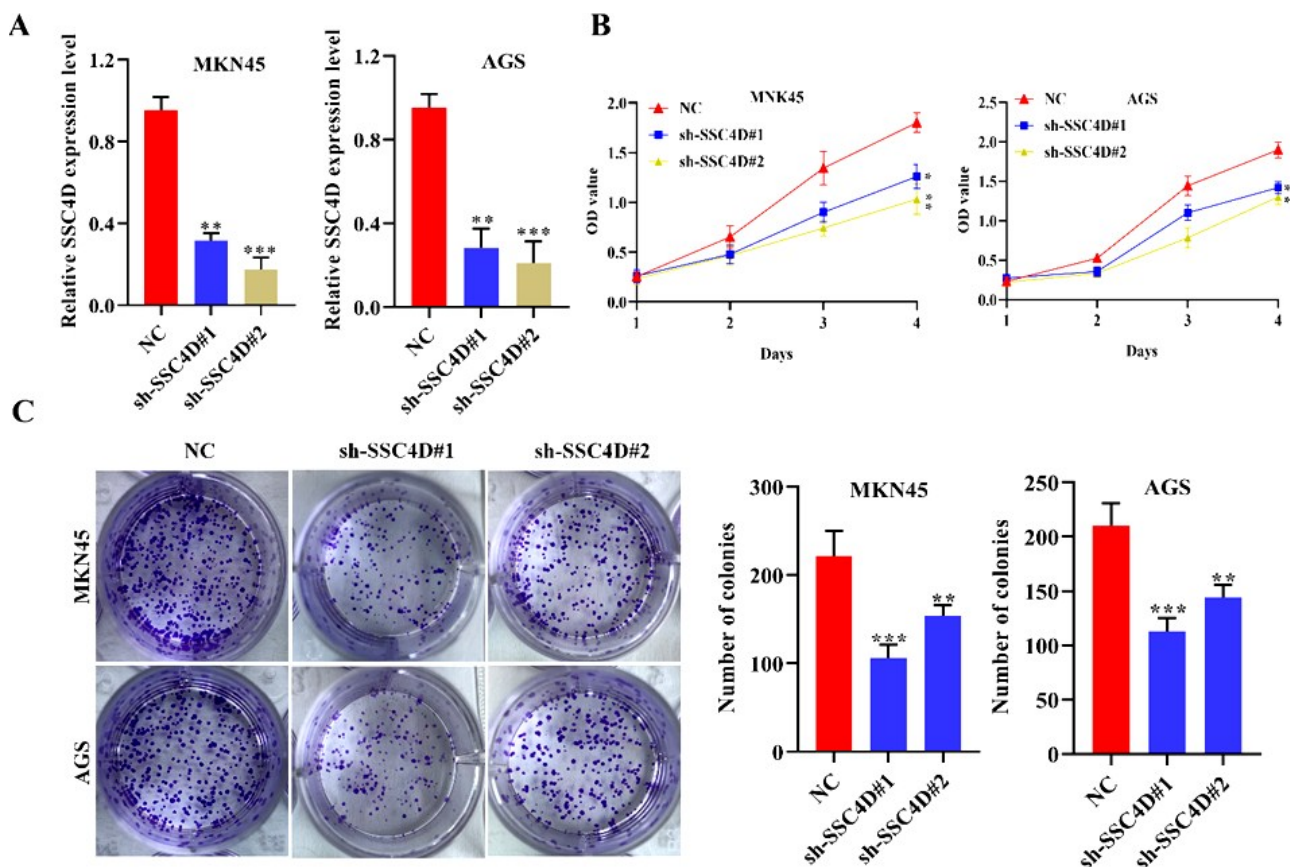


Figure 3. Silencing of SSC4D inhibits MKN45 and AGS cell growth in vitro.

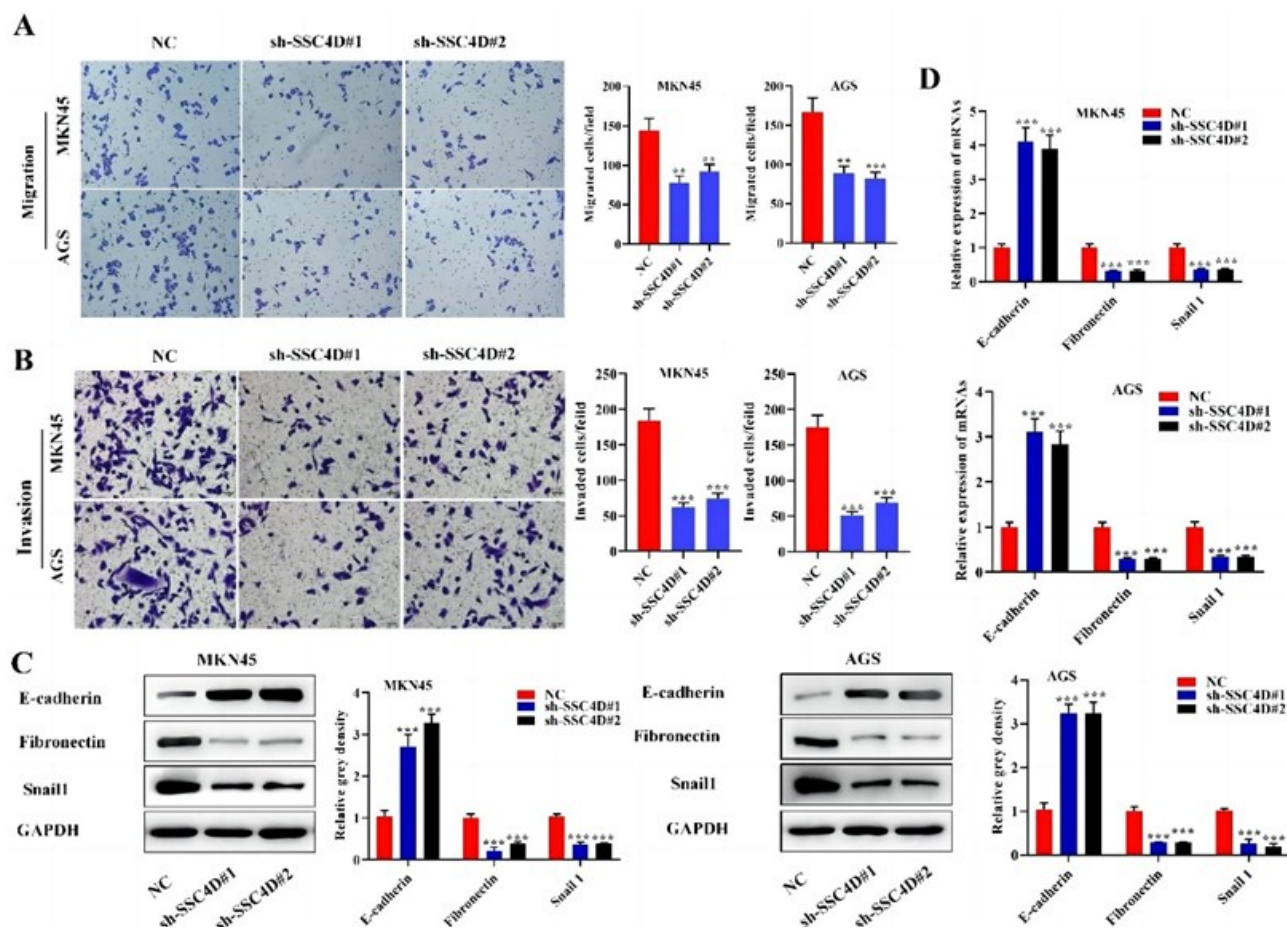


Figure 4. Silencing of SSC4D inhibits AGS and MKN45 cell metastasis in vitro.

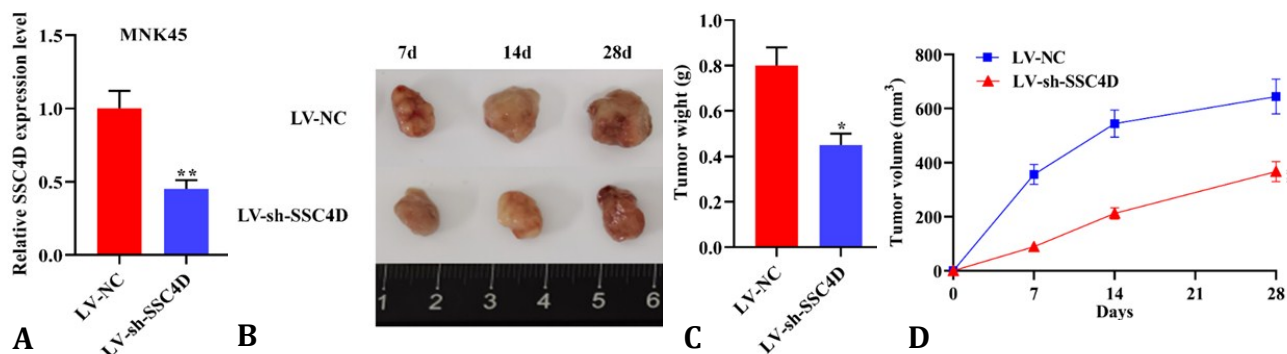


Figure 5. SSC4D silencing suppresses mouse xenograft tumorigenesis.

(A) SSC4D expression in MKN45 cells infected with LV-NC/LV-sh-SSC4D. (B) The tumor images in LV-sh-SSC4D or LV-NC-infected nude mice. (C) Tumor weight in LV-sh-SSC4D or LV-NC-infected nude mice. (D) Tumor volume in LV-sh-SSC4D or LV-NC-infected nude mice. Two-tailed Student's t-test analyzed the statistical difference between two groups. Two way ANOVA compared the statistical difference in tumor volume among different time points, * $p < 0.05$, ** $p < 0.01$.

DISCUSSION

Gastric cancer is a highly lethal malignancy with high prevalence across the world. Patients are

asymptomatic at the early stage and often develop to the advanced stage when diagnosed (26). With the deepening understanding of the molecular biology in GC, the identification of predictive biomarkers and therapeutic targets (27,28). The role of SSC4D is rarely investigated in GC. This work identified the significant upregulation of SSC4D in GC samples and was related to unfavorable prognosis in GC. The diagnosis value of SSC4D was confirmed using a ROC curve with AUC = 0.8685. These results suggested the prognostic and diagnostic value of SSC4D in GC. Additionally, SSC4D expression was found in relation with the tumor size, T Staging, helicobacter pylori infection, and smoking, suggesting its oncogenic function in GC (table 1).

SSC4D, also known as SRCRB4D, with location in the 7q11.23 region. The upregulated expression of

SSC4D is thought to be related to gastric cancer progression, and SSC4D has been found to be enriched in cervical carcinoma, ovarian cancer while differently expressed in desmoplastic small round-cell tumor. Also, SSC4D displays a potential regulatory role on the PI3K-AKT signaling pathway (12-16). Previous study has revealed that SSC4D promotes EMT and cancer malignancy and is related to unfavorable prognosis in esophageal squamous cell carcinoma (16). Moreover, SSC4D has been reported to modulate cancer progression and exhibit potential diagnostic and prognostic roles in different diseases, including cancer (11, 29). SSC4D is indicated to be upregulated in GC based on online cancer databases, whereas, the function of SSC4D is not elucidated in GC. In our work, we not only proved that SSC4D was highly expressed in GC tissues and cells, but also revealed that its upregulation predicted poor prognosis in GC patients. The diagnostic sensitivity and specificity of SSC4D were identified in GC. The expression of SSC4D distinguished normal samples from cancerous samples (figure 1). Moreover, the expression of SSC4D was associated with tumor size, T Staging, helicobacter pylori infection and smoking (table 1). SSC4D silencing was also demonstrated to suppress the proliferation capacity of GC cells and tumorigenesis in tumor-bearing mice (figure 3).

Decreased cell-cell adhesion is found in cancer cells, and is possibly related to cancer invasion and migration (30). We further investigated the biological function of SSC4D on GC cell migration and invasion abilities using the loss-of-function analysis. SSC4D silencing was revealed to exert inhibitory effects on GC cell migration as well as invasiveness. Previous literature has revealed the association of EMT and tumor metastasis, cancer progression can be affected via the EMT process (23-25). Additionally, the SSC4D induced expression changes in EMT-related genes including E-cadherin, Snail (31), as well as Fibronectin (32) were monitored. Our results showed unveiled the SSC4D silencing-induced upregulation of E-cadherin expression as well as downregulation of Fibronectin and Sndil1 in GC cells (figure 4). SSC4D possibly regulated GC cell metastasis via affecting EMT, and these findings were consistent with the published studies. Nevertheless, our study have some limitations. The specific regulatory mechanisms of SSC4D in GC requires further exploration.

In conclusion, our findings for the first time revealed that SSC4D exerts a vital oncogenic role in GC. SSC4D upregulation was identified in GC. SSC4D facilitated cell growth, migration, invasiveness and the EMT process as well as tumor growth in GC. Our findings may shed new light on the targeted therapy in GC and also new biomarkers for GC diagnosis and prognosis.

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Conflicts of interests: The authors have no conflicts of interest to declare.

Ethical consideration: This study was approved by the Ethics Committee of the Haining Traditional Chinese Medicine Hospital (approval numer: [ZJ]-LL001A).

Author contribution: J.Z. and Q.Q. conceptualized the study; J.Z. and T.J. performed the experiments, and collected and analyzed the data. J.Z. and T.J. wrote the original draft, and Q.Q. reviewed and edited the manuscript. All authors read and approved the final manuscript.

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