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RESEARCH PAPER

CHSY1 is upregulated and acts as tumor promotor in gastric cancer through regulating cell proliferation, apoptosis, and migration

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ABSTRACT

Gastric cancer is one of the most frequently diagnosed malignant tumors, with rapid progression and poor prognosis. The role of chondroitin sulfate synthase 1 (CHSY1) in the development and progression of gastric cancer was explored and clarified in this study. The immunohistochemistry analysis of clinical tissue samples as well as data mining of public database showed that CHSY1 was significantly upregulated in gastric cancer and associated with more advanced tumor stage and poorer prognosis. *In vitro* loss-of-function experiments demonstrated the inhibited cell proliferation, colony formation, cell migration, as well as the promoted cell apoptosis by CHSY1 knockdown. Moreover, recovery of CHSY1 expression could attenuate the regulatory effects induced by CHSY1 knockdown. Correspondingly, gastric cancer cells with CHSY1 knockdown showed reduced tumorigenicity and slower tumor growth *in vivo*. In conclusion, this study identified CHSY1 as a tumor promotor in gastric cancer, which may be utilized as a novel indicator of patients' prognosis and therapeutic target for developing more effective drug for GC treatment.

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Gastric cancer; CHSY1; cell proliferation; cell apoptosis; cell migration

Introduction

Gastric cancer is the malignant tumor with the highest morbidity and mortality among digestive system tumors. It is also the fourth most common malignant tumor in the world and the second leading cause of cancer-related death worldwide [1–3]. At present, surgical treatment, drug chemotherapy, and radiotherapy are still the main treatment methods for gastric cancer. However, although surgical treatment has good curative effect for early gastric cancer, patients were commonly diagnosed with the tumor that has advanced to the late stage because of the occult onset and rapid development of gastric cancer, so the prognosis is very poor [4,5]. In recent years, great progress has been made in the basic research and clinical application of gastric cancer. More and more chemotherapeutic drugs are being used in clinic. New treatment strategies such as molecular targeted therapy, tumor immunotherapy, and gene therapy are constantly emerging and updated. However, the long-term survival rate of gastric cancer patients has not been significantly improved [6–9]. Therefore, it is an important way to improve the quality of life and survival rate of gastric cancer patients to carry out multi-angle and multi-level basic research on gastric cancer, further clarifying the molecular mechanism of gastric cancer occurrence and development, and search for highly specific molecular markers or drug treatment targets [10,11].

Chondroitin sulfate (CS) is a sulfated glycosaminoglycan that can be detected in connective tissue of human and other animals [12,13]. Importantly, CS plays an important role in the progression of osteoarthritis [12,14], cardiovascular, cerebrovascular diseases [15], central nervous system-related diseases [16] and other malignant tumors [17]. In addition, CS may exert an anticancer role through immune regulation and inhibition of angiogenesis [18,19]. The biosynthesis of CS requires the participation of various glycosyltransferases, among which chondroitin sulfate synthase 1 (CHSY1) is a key cofactor. On the one hand, CHSY1 can be a vital factor in the regulation

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of joint patterns during normal development [20]. On the other hand, CHSY1 has been identified to be involved in T cell differentiation [21]. Additionally, the carcinogenic effects of CHSY1 in a variety of human cancers, such as soft tissue sarcomas [22], colorectal cancer [23] and hepatocellular carcinoma [24], has been reported. However, the biological function and potential molecular mechanism of CHSY1 in gastric cancer are still puzzling and need to be further explored.

The relationship between the expression of CHSY1 and the progression of gastric cancer was first described in this study. Firstly, the expression level of CHSY1 was distinguished by IHC in gastric cancer tissues and para-carcinoma tissues. Subsequently, knockdown of CHSY1 in gastric cancer cells was used to investigate the effects on proliferation, colony formation, apoptosis, and migration *in vitro*. Furthermore, the mouse xenograft model was used to verify the inhibitory effects of CHSY1 knockdown on gastric cancer *in vivo*. Therefore, CHSY1 was recognized as a key molecule to promote the progression of gastric cancer, which was expected to become a new target for the treatment of gastric cancer.

Material and methods

Cell culture, antibodies, and mice

MGC-803, BGC-823 and AGS cell lines were purchased from BeNa Technology (Hangzhou, Zhejiang, China). MGC-803 cell line was cultured in DMEM-H medium (DMEH-214.5 g/LiterGlucose) with 10% FBS additives and BGC-823 and AGS were cultured in RPMI-1640 medium (Gibco, Rockville, MD, USA) with 10% FBS. All cells were kept in a humid incubator at 37°C with 5% CO_2. All cell medium was changed every 3 days.

CHSY1 (Cat. # orb126811, Biorbyt, San Francisco, CA, USA), p-Akt (Cat. # bs-5193 r, Bioss, Beijing, China), GAPDH (Cat. # AP0063, Bioworld, St. Louis, MN, USA), HRP goat anti-rabbit IgG (Cat. # A0208, Beyotime, Beijing, China) were used in this study. Ki-67 (Cat. # Ab16667), N-cadherin (Cat. # ab18203), MAPK9 (Cat. # ab76125), PIK3CA (Cat. # ab40776) and goat anti-rabbit IgG H&L (HRP) (Cat. # ab6721) were from Abcam (Cambridge, MA, USA). Vimentin (Cat. # 3932), Snail (Cat. # 3879S), and Cyclin D1 (Cat. # 2978) and Akt (Cat. # 4685) were from CST (Danvers, MA, USA).

Four-week BALB/c nude mice were purchased from Shanghai Lingchang (Shanghai, China) and grown in SPF conditions. Animal experiments were approved by the Institutional Animal Care and Use Committee of Jilin University.

Immunohistochemistry (IHC) and Ki-67 immunostaining

The resected surgical tissue samples including 86 tumor and 124 para-carcinoma tissues (collected from 2013.1 to 2015.8) were fixed with 10% formalin and embedded with paraffin as tissue microarray. Related information of all gastric cancer patients included was collected and written informed consents were collected as well. These experiments were approved by the Ethics Committee of Jilin University.

For immunohistochemistry, in short, tissue microarray was dehydrated with xylene and rehydrated with alcohol, antigen repaired with sodium citrate buffer at 120°C. Then, the tissue microarray was blocked with 3% H_2O_2, CHSY1 antibody (1:300) were added for incubating overnight at 4°C. Then, the tissue microarray was further stained with the second antibody HRP goat anti-rabbit IgG (1:3000) for 2 h at room temperature. The expression of CHSY1 was visualized by using DAB horseradish peroxidase color development Kit. All slides were viewed with ImageScope and CaseViewer. IHC scoring was accomplished by two independent pathologists. Staining intensity was classified as follows: 0, no staining; 1, light yellow; 2, brown yellow; 3, dark brown. The staining percentage scored as follows: 1, 1%–24%; 2, 25%–49%; 3, 50%–74%; 4, 75%–100%. IHC scoring was determined by staining intensity score and the staining percentage score, median of which was used to divide all samples into high/low CHSY1 expression groups.

For mice tumor Ki-67 immunostaining assay, 5 μm tumor tissue slides were prepared. Primary antibody Ki-67 (1:200) were added and incubated overnight at 4°C. Next, slides were stained by the goat anti-rabbit IgG H&L (HRP) (1:400) for 2 h at

room temperature. Slides were visualized by hematoxylin and eosin staining.

Plasmid constructs and cell transfection

Escherichia coli

Three shRNA sequences [shCHSY1, targeting 5′-GCACAAAGAACCCAAAGATAA-3′ (shCHSY-1), 5′-TGAGAATTACGAGCAGAACAA-3′ (shCHSY-2), 5′-GCAAATACAGCAACACAGAAA-3′ (shCHSY-3), respectively] of CHSY1 were designed and prepared by Shanghai Bioscienceres, Co., Ltd (Shanghai, China). cDNAs were synthesized and double stranded DNA oligo with the sequences were obtained and linearized, then the DNA sequences were cloned into linearized victor BR-V108 (Shanghai YBR Bioscienceres, Co., Ltd. Shanghai, China). Ligation products were transfected TOP10 *Escherichia coli* competent cells and DNA sequencing analysis was used to confirm the vector expression. Plasmid was extracted with EndoFree maxi plasmid kit (Tiangen, Beijing, China). Thermo Nanodrop 2000 (Waltham, MA, USA) was used to determine the concentration of plasmid. Then, lentivirus expressing shCHSY1 were constructed. Similarly, lentivirus expressing a random sequence was prepared as negative control and named as shCtrl. For overexpressing CHSY1, the CHSY1 construct was generated by subcloning human CHSY1 cDNA into vector BR-V214 (Shanghai YBR Bioscienceres, Co., Ltd. Shanghai, China) (CHSY1 group) while an unloaded lentivirus was used as negative control (control group).

For cell transfection, AGS and MGC-803 cells were infected with 40 μL 1×10^8 TU/mL prepared lentivirus along with ENI.S and Polybrene additives. All cells were cultured for 72 h. Cell infection efficiency was estimated by microscopic fluorescence.

Real-time PCR

Total RNAs of gastric cancer cells with or without CHSY1 knockdown were extracted by trizol (Invitrogen, Carlsbad, CA, USA). The quality of total RNA was evaluated by Thermo Fisher Scientific Nanodrop 2000/2000 C spectrophotometer (Waltham, MA, USA). cDNAs were synthetized with 2.0 μg total RNA using Promega M-MLV Kit (Heidelberg, Germany). Quantitative real-time PCR was conducted with Vazyme SYBR Green mastermixs Kit (Nangjing, Jiangsu, China) and Biosystems 7500 Sequence Detection system was applied. The PCR reaction conditions were as follows: 95°C for 30 s, 95°C for 5 s, 60°C for 30 s, 95°C for 15 s, 60°C for 30 s, and 95°C for 15 s, for 40 cycles. The forward and reverse primers for CHSY1 were AGTGGGTGGCTTTGATGTTTC and AGGATGGTGGACGTGGACTA (5′-3′), respectively; that for GAPDH were TGACTTCAACAGCGACACCCA and CACCCTGTTGCTGTAGCCAAA (5′-3′), respectively. GAPDH was used as inner control.

Western Blot

First, total proteins were collected after the AGS and MGC-803 cells were fully lysed by cold RIPA buffer (Millipore, Temecula, CA, USA) and concentration of the protein was detected by a BCA Protein Assay Kit (HyClone-Pierce, Logan, UT, USA). 20 μg per lane protein samples were separated by 10% SDS-PAGE (Invitrogen, Carlsbad, CA, USA), then all samples were moved onto PVDF membranes. The PVDF membranes were blocked with TBST solution of 5% degreased milk at room temperature for 1 h. The PVDF membranes were incubated primary antibodies including CHSY1 (1:500), N-cadherin (1:1000), Vimentin (1:2000), Snail (1:1000) and GAPDH (1:3000) antibodies at 4°C overnight, then stained with HRP goat anti-rabbit IgG (1:3000) for 2 h at room temperature. The blots were analyzed by enhanced chemiluminescence (ECL) (Amersham, Chicago, IL, USA).

Celigo cell counting assay

The transfected cells were seeded into 96-well plates (1,000 cells/well) in triplicate. After culturing in a 5% CO_2 incubator at 37°C for another day, cell images were captured using Celigo image cytometer (Nexcelom Bioscience, Lawrence, MA, USA) according to the instructions of the manufacturer once a day for 5 days.

MTT assay

The transfected cells were seeded with 2000 cells/well in triplicate and cultured for 5 days. We added 20 μL MTT (5 mg/mL, GenView, El Monte, CA, USA) to each well after culturing for 24, 48, 72, 96 h and 120, respectively. Cells were continuously cultured for 4 h, 100 μL of dimethyl sulfoxide (Shanghai ShiYi Co., Ltd. Shanghai, China) was added. The OD value at 490 nm wavelength was determined using Thermo Fisher enzyme microplate reader (Waltham, MA, USA). The inhibitory rate of cell proliferation was calculated as: [(OD490 value of shCtrl+Control cells at day 5) – (OD490 value of shCHSY1+ CHSY1 cells at day 5)]/(OD490 value of shCtrl cells at day 5).

Cell apoptosis

Cells (1×10^3 cells per well) were inoculated in 6-well plates in triplicate and further cultured till the cell confluence reached 85%. Cells were collected, after centrifuged at 1300 × g, cells were resuspended and Annexin V-APC (10 μL, eBioscience, San Diego, CA, USA) was added for staining 10 min without light. Apoptosis analyses was measured using FACSCalibur (BD Biosciences, San Jose, CA, USA). Notably, the fluorescence of the GFP tag on lentivirus was detected and used as the Y-axis. Therefore, the cells that are not infected with shCHSY1 or shCtrl were excluded from the detection of apoptotic assay.

Colony formation assay

Cells in logarithmic growth phase were inoculated in a 6-well plate (1000 cells per well) in triplicate and further cultured for 8 days. Fluorescent images were photographed under a fluorescence microscope (Olympus, Tokyo, Japan). The cells were then fixed with 4% paraformaldehyde and stained by Giemsa. Photographs of colonies were collected with a digital camera and clones contain more than 50 cells were counted.

Wound-healing assay

0.1 mL (5×10^5 cells/well) transfected AGS and MGC-803 cells were plated into a 96-well dish for culturing. Wounds across the cell layers were formed via a wounding replicator. Photographs were taken by a fluorescence microscope at 0 h, 24 h and 48 h after scratching. The width of the wound was measured at the start of the experiment (0 h) and at the indicated time point (24 h, 48 h). Cell migration rates of each group were calculated [width (0 h) – width (24 h or 48 h)]/width (0 h).

Human apoptosis antibody array

Human apoptosis signaling pathway was performed using Human Apoptosis Antibody Array (R&D Systems, Minneapolis, MN, USA) following the manufacturer's instructions. Briefly, transfected MGC-803 cells (shCtrl and shCHSY1) were solubilized in lysis buffer for extracting total proteins. After centrifugation at 12,000 g for 5 minutes at 4°C, the protein concentration was detected by BCA Protein Assay Kit (HyClone-Pierce, Logan, UT, USA). 20 μg total proteins were added into the antibody-coated Human Apoptosis Antibody Array membranes (R&D Systems, Minneapolis, MN, USA) for incubating overnight at 4°C. The membranes were then incubated with HRP linked Streptavidin conjugate. Membrane intensity was acquired using enhanced chemiluminescence (ECL) (Amersham, Chicago, IL, USA).

Animal experiments and fluorescence imaging

For tumorigenicity, each female nude mice received a subcutaneously injection of 0.2 mL (2×10^7 cells/mL) transfected MGC-803 (shCtrl or shCHSY1) cells with 5 mice in shCtrl group and 5 in shCHSY1 group. Two weeks post injection, we continuously collected the data of mice's weight and the tumor sizes 2 times per week. The volume of tumor was calculated as $0.5 \times L \times W^2$ (W, width at the widest point; L, perpendicular width). One day before sacrificing the animal models, all mice were anaesthetized by

intraperitoneal injection of 0.7% Sodium Pentobarbital (10 μL/g, Sigma, St Louis, MO, USA). Anaesthetized mice were put on the IVIS Spectrum Imaging System (Perkin Elmer, Waltham, MA, USA) for fluorescence imaging. Finally, mice were sacrificed and tumors were extracted, weighed, and imaged.

Statistical analyses

Continuous experimental data in our study were shown as mean ± SD and categorical variables were expressed as percentages. All statistical analysis was done by SPSS 22.0 (IBM, Chicago, IL, USA). Student's *t* test was used to determine the significance between groups. One-way ANOVA followed by Bonferroni's post hoc test was used for determining the significance among multiple groups. For patients' characteristics and CHSY1 expression, Mann–Whitney U analysis and Spearman correlation analysis was performed. A value of $P < 0.05$ was considered statistically significant.

Results

CHSY1 was upregulated in gastric cancer

For the sake of exploring the role played by CHSY1 in gastric cancer, its expression in tumors collected from patients with gastric cancer was detected using immunohistochemical (IHC) analysis and compared with para-carcinoma ones. Based on the median of IHC scores of all tissue samples, they were divided into high expression group and low expression group. As illustrated by Figure 1(a) and Table 1, the outcomes of IHC showed that CHSY1 was significantly upregulated in gastric cancer tissues. Further comparison indicated the relatively higher expression of CHSY1 in

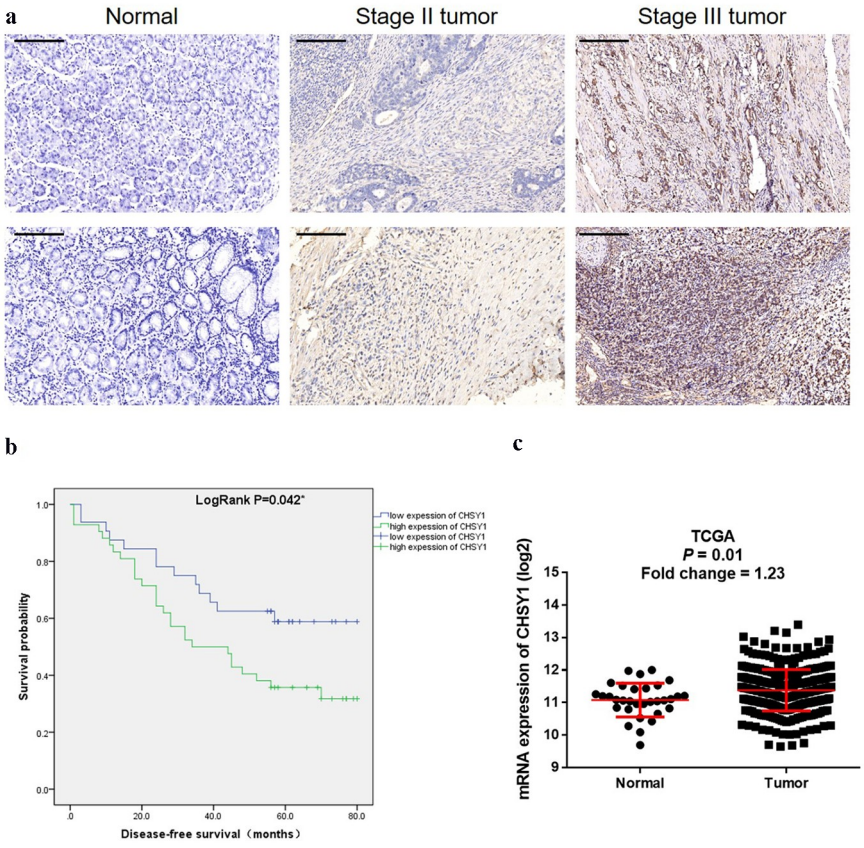

Figure 1. CHSY1 was upregulated in gastric cancer. (a) The expression of CHSY1 in tumor tissues of gastric cancer was detected by IHC and compared with para-carcinoma tissues, showing that CHSY1 was upregulated in gastric cancer and associated with tumor stage (scale bar = 50 μm). (b) Kaplan-Meier survival analysis showed that CHSY1 high expression was significantly associated with relatively poor prognosis. (c) Data mining of TCGA database showed that expression of CHSY1 is relatively higher in gastric cancer tissues compared with para-carcinoma tissues.

Table 1. Expression patterns of CHSY1 in gastric cancer tissues and para-carcinoma tissues revealed in immunohistochemistry analysis.

	Tumor tissue		Para-carcinoma tissue	
CHSY1 expression	Cases	Percentage	Cases	Percentage
Low	39	45.3%	124	100%
High	47	54.7%	0	-

$P < 0.001$

tumor tissues with more advanced stage (Figure 1(a)). More importantly, a significant correlation was discovered between high expression of CHSY1 and relatively short survival period, as well as poor prognosis ($P = 0.042$, Figure 1(b)).

Table 2. Relationship between CHSY1 expression and tumor characteristics in patients with gastric cancer.

		CHSY1 expression		
Features	No. of patients	low	high	*P* value
All patients	86	39	47	
Age (years)				0.203
<64	42	22	20	
≥64	44	17	27	
Gender				0.699
Male	57	25	32	
Female	29	14	15	
T Infiltrate				0.212
T1	6	3	3	
T2	11	8	3	
T3	55	22	33	
T4	14	6	8	
lymphatic metastasis (N)				0.459
N0	12	7	5	
N1	13	8	5	
N2	19	5	14	
N3	42	19	23	
AJCC Stage				0.044
I	5	2	3	
II	19	14	5	
III	61	22	39	
IV	1	1	0	
Tumor size				0.948
<5 cm	38	17	21	
≥5 cm	44	20	24	
Vessel carcinoma embolus				<0.001
0	17	12	5	
1	48	11	37	
Expression of CD34				0.184
no	11	7	4	
yes	30	12	18	
Expression of EGFR				0.710
no	62	30	32	
yes	14	6	8	
Expression of VEGF				0.556
no	39	20	19	
yes	36	16	20	
Expression of Her2				0.122
no	60	26	34	
yes	17	11	6	

Consistently, analysis of data in The Cancer Genome Atlas (TCGA) also revealed the upregulated expression of CHSY1 in gastric cancer tissues (Fold change 1.23, $P = 0.01$, Figure 1(c)). Next, chi-square test was performed to assess the association between CHSY1 expression and tumor characteristics of the patients (Table 2). It was demonstrated that high expression of CHSY1 could predict more advanced tumor stage, and higher risk of vessel carcinoma embolus. The correlation was also confirmed by Spearman rank correlation analysis (Table 3). Collectively, these results suggested that CHSY1 may have critical functions in the gastric cancer progression.

CHSY1 knockdown suppressed cell growth and promoted cell apoptosis

Given the above results, we next constructed CHSY1 knockdown cell lines based on AGS and MGC-803 cells through transfection of lentivirus expressing shRNAs for silencing CHSY1. QPCR and western blotting were utilized to measure the knockdown efficiencies of CHSY1 in AGS and MGC-803 cells. Based on the combined evaluation of qPCR (Figure 2(a)) and western blotting (Figure 2(b)), shCHSY-1 and shCHSY-3 with relatively better knockdown efficiencies were selected for further experiments.

For investigating the regulatory effects of CHSY1 on development of gastric cancer, cell proliferation, colony formation and cell apoptosis were detected in AGS and MGC-803 cells with or without CHSY1 knockdown. We performed celigo cell couting assay for detecting cell proliferation and found that CHSY1 knockdown significantly inhibited cell growth of AGS

Table 3. Relationship between CHSY1 expression and tumor characteristics in patients with gastric cancer analyzed by Spearman rank correlation analysis.

Tumor characteristics	index	
AJCC stage	Spearman correlation	0.218
	Significance (two tailed)	0.043
	n	86
Vessel carcinoma embolus	Spearman correlation	0.443
	Significance (two tailed)	<0.001
	n	65

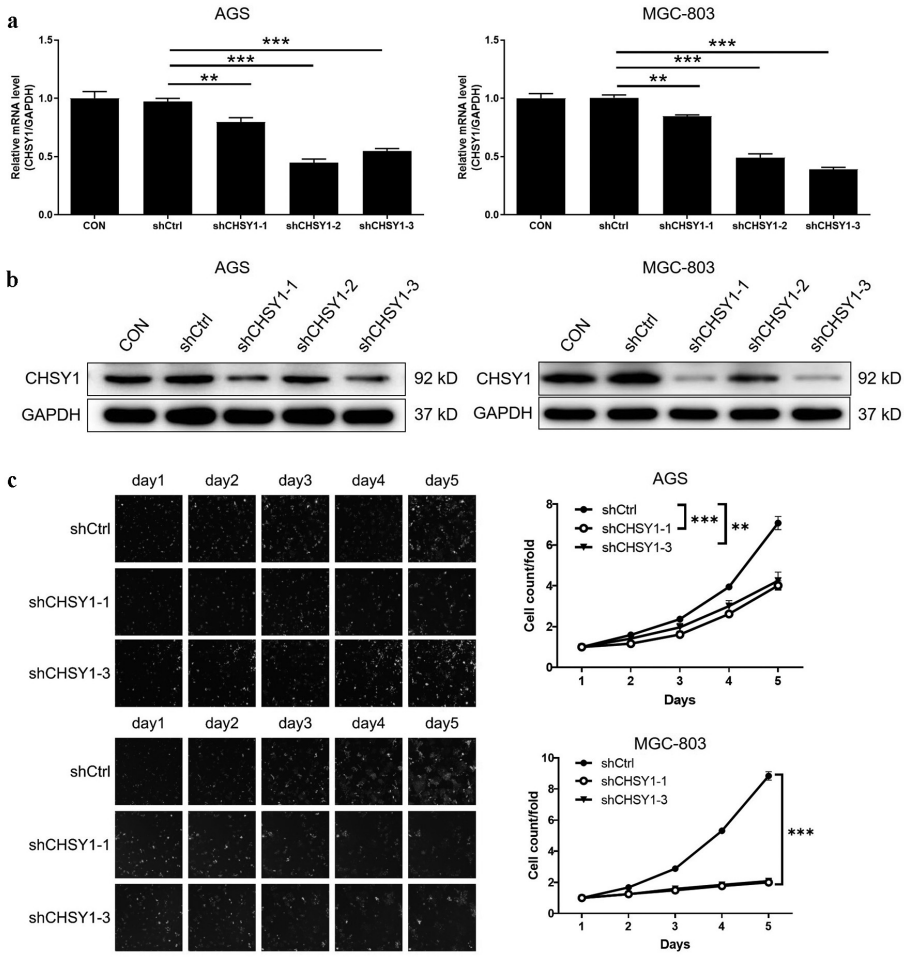

Figure 2. Construction of gastric cancer cell models with CHSY1 knockdown. (a) qPCR was utilized to detect the expression of CHSY1 in AGS and MGC-803 cells for evaluating the knockdown efficiency. (b) The successful knockdown of CHSY1 in AGS and MGC-803 cells was further verified by detecting its protein level through western blotting. (c) Celigo cell counting assay was performed to detect cell proliferation of AGS and MGC-803 cells with or without CHSY1 knockdown and showed that knockdown of CHSY1 significantly inhibited cell proliferation. The data was shown as mean ± SD. $^{**}P < 0.01$, $^{***}P < 0.001$.

and MGC-803 cells ($P < 0.01$, Figure 2(c)). The measurement of colony formation also showed the significantly decreased capability of AGS and MGC-803 cells to form colonies in CHSY1 knockdown groups ($P < 0.001$, Figure 3(a)). As anticipated, the detection of cell apoptosis demonstrated the significantly promoted apoptosis in both cell lines by CHSY1 knockdown (> 2-fold promotion, $P < 0.001$, Figure 3(b)). To identify the potential mechanisms by which CHSY1 knockdown regulates cell apoptosis, Human Apoptosis Antibody Array was performed to screen differentially expressed apoptosis-related proteins in shCHSY1 (shCHSY-1) group *vs.* shCtrl group of MGC-803 cells. As shown in Figure 4 (a), the outcomes showed the CHSY1 knockdown induced downregulation of Bcl-2, clAP-2, sTNF-R1, TNF-α and XIAP ($P < 0.05$). Moreover, we further showed that infection of CHSY1-overexpressing lentivirus in shCHSY1 cells could alleviate its inhibitory effects on cell proliferation (Figure 4(b)). In summary, these results illustrated the potential promotion effects of CHSY1 on development of gastric cancer.

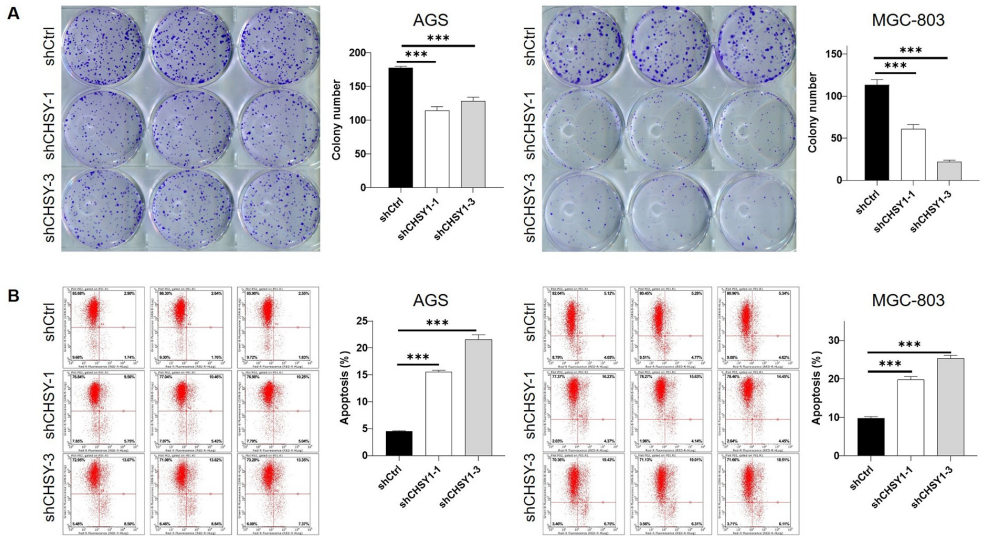

Figure 3. CHSY1 knockdown inhibited colony formation and promoted cell apoptosis arrest in gastric cancer cells. (a) The ability of AGS and MGC-803 cells with or without CHSY1 knockdown to form colonies was examined and compared by colony formation assay. (b) The results of flow cytometry (single staining by Annexin V-APC) demonstrated that knockdown of CHSY1 obviously promoted cell apoptosis of AGS and MGC-803 cells. The data was shown as mean ± SD. ***$P < 0.001$.

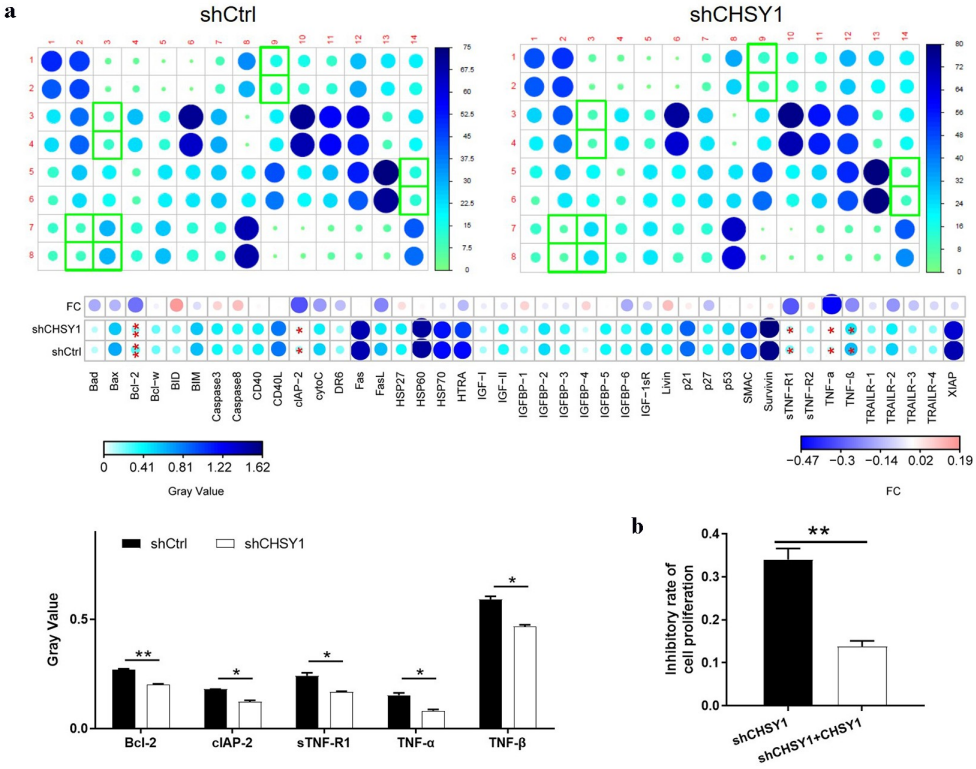

Figure 4. CHSY1 knockdown promoted cell apoptosis through regulation apoptosis-related proteins. (a) Human Apoptosis Antibody Array was performed to identify the differential expression of apoptosis related proteins between shCtrl and shCHSY1 MGC-803 cells and showed the downregulation of Bcl-2, cIAP-2, sTNF-R1, TNF-α and XIAP in shCHSY1 group. (b) After overexpressing CHSY1 in CHSY1 knockdown MGC-803 cells, the inhibitory rate of shCHSY1+ CHSY1 cells was detected and compared with shCHSY1 cells, showing that the recovery expression of CHSY1 could alleviate the inhibition of cell proliferation by CHSY1 knockdown. The data was shown as mean ± SD. *$P < 0.05$, **$P < 0.01$.

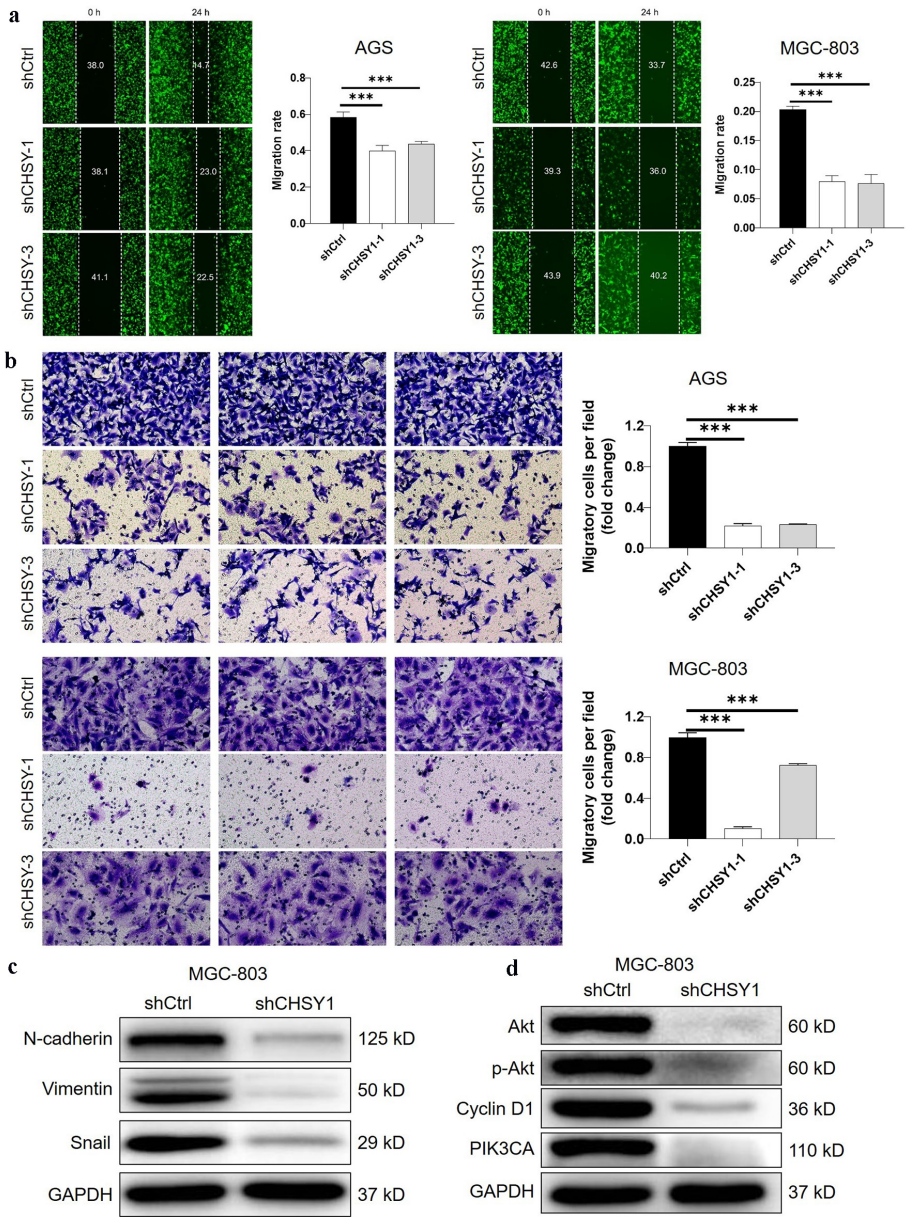

Figure 5. CHSY1 knockdown inhibited cell migration and expression of EMT-related proteins. (a, b) Wound-healing assay (a) and transwell assay (b) were used to detect cell migration rate and showed that cell migration of AGS and MGC-803 cells was significantly suppressed by CHSY1 knockdown. (c) The results of western blotting clarified that knockdown of CHSY1 could clearly downregulated expression of EMT-related proteins including N-cadherin, Vimentin and Snail. (d) The results of western blotting showed the downregulation of Akt, p-Akt, Cyclin D1 and PIK3CA in shCHSY1 MGC-803 cells. The representative images were randomly selected from at least 3 independent experiments. The data was shown as mean ± SD. ***$P < 0.001$.

CHSY1 knockdown inhibited cell migration and expression of EMT-related proteins

Subsequently, the functions of CHSY1 in motility of gastric cancer cells as well as its potential role in tumor metastasis were examined through wound-healing and transwell assays. It was demonstrated that CHSY1 knockdown significantly suppresses cell migration of AGS and MGC-803 cells ($P < 0.01$, Figure 5(a-b)). Subsequently, the effects of CHSY1 knockdown on expression of epithelial-mesenchymal transition (EMT)-related proteins, showing the downregulation of N-cadherin, Vimentin and

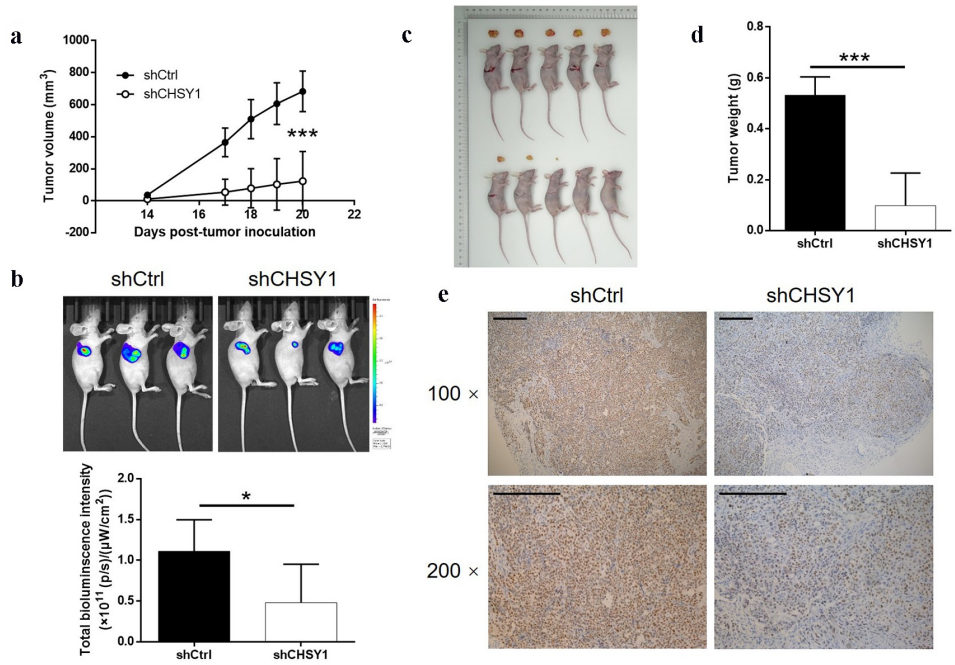

Figure 6. CHSY1 knockdown inhibited tumor growth of gastric cancer *in vivo*. (a) Tumor volume of tumors was measured and calculated throughout the culture of animal models and showed the obviously slower growth of xenografts in shCHSY1 group. (b) The fluorescence intensity obtained by *in vivo* imaging showed apparently smaller tumors in shCHSY1 group. (c) The photos of the removed tumors were obtained after sacrificing the animals. (d) The weight of the tumors was measured, which showed that tumors in shCHSY1 group were lighter. (e) The IHC analysis of Ki-67 expression in tumors showed obvious higher levels in shCtrl group (scale bar = 50 μm). The representative images were randomly selected from at least 3 independent experiments. The data was shown as mean ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Snail in shCHSY1 group (Figure 5(c)). After verifying the functions exerted by CHSY1 in the growth and metastasis of gastric cancer, the molecular mechanism was also preliminarily investigated. As shown in Figure 5(d), expression of several factors including Akt, p-Akt, Cyclin D1 and PIK3CA, which were well known to play critical roles in human cancers, were detected by western blotting and showed obvious downregulation in shCHSY1 group of MGC-803 cells.

CHSY1 knockdown inhibited tumor growth of gastric cancer *in vivo*

To further investigate the role of CHSY1 in gastric cancer *in vivo*, MGC-803 cells with or without CHSY1 knockdown were subcutaneously injected into mice for constructing mice xenograft models. Throughout the culture of animal models, the growth of tumors was observed and the volumes were calculated based on the tumor size. We found that CHSY1 knockdown could obviously slow down the rate of tumor growth *in vivo* ($P < 0.001$, Figure 6(a)). Consistently, the suppression of tumor growth by CHSY1 knockdown was also visualized by bioluminescence imaging which was facilitated by injection of D-Luciferin ($P < 0.05$, Figure 6(b)). After sacrificing the animals, the photos of tumors were obtained and weights of tumors were measured, both indicating smaller tumors in shCHSY1 group ($P < 0.01$, Figure 6c and6d)). Besides, Ki-67, which was considered as representation of tumor growth, was also detected in the removed tumors and exhibited apparently downregulation in shCHSY1 group (Figure 6(e)). Altogether, the results suggested that knockdown of CHSY1 could restrain tumor growth *in vivo*.

Discussion

The development and metastasis of gastric cancer is a complex process, which involves multiple genes and lots of signaling pathways. Currently, its molecular mechanism has not been clearly

elucidated [25]. Recently, the role played by some genes or pathways in gastric cancer development has been explored and revealed [26,27]. For example, HER2 has been well-documented to be a tumor promotor, as well as a therapeutic target, in the development of gastric cancer that could promote gastric cancer through regulating cell proliferation, migration and infiltration [28–30]. c-Met is another well-known tumor promotor of gastric cancer that was associated to short survival of gastric cancer patients [31–33]. On the other hand, Wnt/β-catenin signaling pathway could regulate differentiation and metastasis of gastric cancer through targeting downstream molecules such as HMGA1 or SOX17 [34–36]. Although the research on molecular mechanism of gastric cancer has become a hot topic in the field of gastric cancer research, its research results have not yet made revolutionary progress in the treatment of gastric cancer. Therefore, it is of great significance to continuously mine the molecular mechanism of gastric cancer and explore the key molecules in the progress of gastric cancer for improving the prognosis of gastric cancer patients.

CS is widely distributed in extracellular matrix and cell membrane of various tissues [34–36], which is closely associated with the numerous biological behaviors such as brain neural network development, cell division and tissue morphology [37]. Moreover, CS could play a vital role in inhibiting the regeneration of axons after spinal cord injury [38], preventing abnormal cardiac remodeling [39] and other physiological functions. Interestingly, the regulatory role of CS in some malignant tumors were identified recently. CS mediated N-cadherin/β-catenin signal transduction is associated with basal-like breast cancer cell invasion and may be a promising target for the treatment of this disease [40]. In addition, the content of CS is related to the cell proliferation activity and prognosis of glioma [4142]. Based on all the above results, it was reasonable to deduce that CHSY1 may play some roles in the development of malignant tumors. In fact, several pieces of work have proved its regulatory functions in a few types of human cancers. For example, Jiang *et al.* identified CHSY1 as a molecular target of LINC01094/miR-224-5p axis in the regulation of clear cell renal cell carcinoma (ccRCC), which could promote the growth and metastasis of cancer cells [41]. It was also demonstrated that CHSY1 could selectively regulate PDGFRA activation and PDGF-induced signaling by stabilizing PDGFRA protein in glioma cells, thus promoting glioma development and predicting poor prognosis [43]. However, to the best of our knowledge, the function of CHSY1 in development of gastric cancer has not been elucidated.

Here, we revealed that the expression of CHSY1 was upregulated in gastric cancer tissue and positively correlated with tumor stage, and the risk of vessel carcinoma embolus. Subsequently, knockdown of CHSY1 significantly interfered with the cell viability, colony growth and induced apoptosis of AGS and MGC-803 cells. In addition, *in vivo* experiments using a mouse xenograft model of MGC-803 cells showed inhibition of tumor growth, which was consistent with the results *in vitro*. Further analysis demonstrated that CHSY1 regulates cell migration through EMT. Therefore, CHSY1 may play a role in promoting gastric cancer and is expected to become a new target for the treatment of gastric cancer.

Akt (also known as protein kinase B, PKB) plays an important role in regulating cell growth, proliferation, survival and metabolism [44]. It has also been reported as a target for the treatment of gastric cancer [45,46]. Accordingly, we found that knockdown of CHSY1 could downregulate the expression of total Akt and its phosphorylation. Cyclin D1, which is an important regulator in cell cycle and cell proliferation [47], have been demonstrated to be highly expressed in gastric cancer and is of great significance for many aspects of cancer research and prognosis judgment [474849]. Consistently, our results showed significantly downregulation of Cyclin D1 upon knockdown of CHSY1, indicating the potential mechanism of CHSY1 knockdown to inhibit gastric cancer. In addition, PIK3CA has been identified as oncogene in various human cancers [50–52], was also found to be downregulated in shCHSY1 group. Despite of these results, the underlying mechanism of the regulatory effects of CHSY1 on gastric cancer is still not clear and would be the focus of our future work.

In conclusion, our studies identified CHSY1 as a tumor promotor of gastric cancer, which presented high expression in gastric cancer and exert its functions through regulating growth and migration of cells, formation of colonies, and cell apoptosis. Therefore, CHSY1 may be considered as a novel indicator for gastric cancer patients' prognosis and its knockdown may serve as a promising strategy of gastric cancer treatment.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Author contributions

J.Z. designed this program. J.L. operated the cell and animal experiments. D.W., T.L. and Z.M. conducted the data collection and analysis. Y.L. produced the manuscript that was checked by J.Z. and J.L. All the authors have confirmed the submission of this manuscript.

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