Ceramide synthase 6 predicts the prognosis of human gastric cancer: It functions as an oncoprotein by dysregulating the SOCS2/JAK2/STAT3 pathway

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Although gastric cancer (GC) is one of the most common cancers, knowledge of its development, and carcinogenesis is limited. The present study explored the involvement of ceramide synthase 6 (CERS6) in GC carcinogenesis and prognosis. RT-PCR, immunoblotting, and immunohistochemistry were used to examine the expression of CERS6. Transfection and small hairpin RNA technology were used to investigate the effect of CERS6 manipulation on cell proliferation and spread as well as the underlying mechanism. Moreover, xenograft proliferation was employed to explore the influence of CERS6 on tumor growth in animals. It was found that overexpression of CERS6 was significantly correlated with several clinicopathologic parameters and poor disease-free survival. The overexpression and silencing of CERS6 in GC cells facilitated and suppressed cell proliferation and spread as well as xenograft proliferation, respectively. Mechanistic studies further revealed that CERS6 influenced cell proliferation and spread by regulating cell cycle control and metastasis-related protein through the SOCS2/JAK2/STAT3 signaling pathway. Collectively, this study suggests that CERS6 overexpression could be a useful
1 | INTRODUCTION

The mortality of gastric cancer (GC) remains the third highest among all cancers. In Taiwan, according to a 2012 government report, the incidence of GC ranked seventh among all types of cancer, with more than 2000 people dying of it annually. Because of a lack of understanding regarding the pathogenesis of GC and the lack of a specific targeted gene therapy, chemotherapy, and surgical techniques do not satisfactorily enhance the survival rate of patients with GC.

Some studies have indicated that genetic alteration may play a crucial role in the development of GC and carcinogenesis. Several genetic alterations which can be used as prognostic biomarkers include oncoproteins such as protein kinase CK2, VAV3, and SOX4, as found in our laboratory, and tumor suppressors such as miR-429 and miR-1225-5p, as found in other laboratories.

Furthermore, with the development of modern technologies, various novel biomarkers had been identified that appear to possess prognostic value. Novel biomarkers for determining the progression and prognosis of GC focus on targeting epigenetic alterations and genetic polymorphisms. The hypermethylation of various genes, such as cadherin 1 and E-cadherin, has been identified to be associated with the prognosis of GC. Various studies have demonstrated that the presence of numerous SNPs is associated with increased GC susceptibility; for example, VEGF and NFκB1 gene SNPs have been found to be associated with the risk of GC.

Sphingolipid is a type of membrane lipid and signal transduction molecule in eukaryotic cells that regulates cellular process related to the development, progression, and metastasis of cancer as well as to drug resistance. Ceramide is a type of sphingolipid synthesized by six transmembrane proteins referred to as ceramide synthases. Various types of ceramide synthases synthesize various types of ceramides with differing chain lengths. Ceramide is crucial to the cellular regulation of apoptosis and cell cycle arrest, and ceramide-related research has received substantial attention in cancer studies. Previously, researchers believed that ceramide could inhibit the growth of cancer cells and cause their death; however, this view is now considered not entirely correct for several reasons. For example, C16-ceramide produced from ceramide synthase 6 (CERS6) was found to be related to cancer cell growth; conversely, C18-ceramide produced from CERS1 has been shown to facilitate cancer cell death.

A recent study determined that higher amounts of C16-ceramide were related to positive lymph node status in patients with breast cancer, indicating the metastatic potential of C16-ceramide in the clinic. Another study showed that the amounts of both CERS2 and CERS6 mRNA increased in breast cancer tumors. The aforementioned studies have shown that ceramide of a specific chain length may play a prosurvival role in cancer pathogenesis.

Among all ceramide synthases, CERS6 is the most recently identified: its clinical importance has gained increasing attention in recent years, with reports suggesting that CERS6 may contribute to tumor cell survival. Senkal et al have indicated that increasing expression of wild-type CERS6 facilitated xenograft proliferation of human squamous cell cancer cells implanted in SCID mice. Conversely, the use of small interfering RNA to knock down the expression of CERS6 in human squamous cell cancer cells induced apoptosis.

However, the way in which CERS6 regulates tumor cell growth remains unclear. Few studies have examined the relationship between ceramide synthases and prognosis. According to Wang et al, the expression of CERS2 is related to the development and progression of bladder cancer in humans; in addition, CERS2 can serve as a prognostic biomarker for bladder cancer. Moreover, CERS6 mRNA and protein have been found to be overexpressed in breast cancer, head and neck cancer, and lung cancers.

Our recent literature review on the relationship between CERS6 and cancer prognosis indicates that only one study, by Suzuki et al, determined that CERS6 is overexpressed in lung cancer tissues and cells and that CERS6 could serve as a prognostic biomarker for lung cancer. The expression of CERS6 in GC, the correlation between CERS6 expression and clinicopathologic parameters, and the importance of CERS6 expression in GC prognosis remain unclear. The objective of this research is to study the expression of CERS6 in normal and GC tissues and cell lines, to study the role of CERS6 in GC tumorigenicity, and to evaluate the possibility of CERS6 as a target for GC chemotherapy and also as a prognostic biomarker for GC.

2 | MATERIALS AND METHODS

2.1 | Cell culture

The human normal gastric cell line (Hs738.St/Int) was obtained from the American Type Culture Collection (Manassas, VA). GC cell lines (AGS, NCI-N87, TMC-1, and TSGH 9201) were obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan). GC cell lines (SK-GT-2 and HGC-27) were obtained from the European Collection of Cell Cultures (Salisbury, UK). The GC cell line (23132/87) was obtained from Creative Bioarray (Shirley, NY). All cell lines were authenticated by short tandem repeat profiling and used within 6 months. Cells were cultured in DMEM (Hs738.St/Int), F-12 K (AGS), RPMI-1640 (NCI-N87, TMC-1, TSGH 9201, SK-GT-2, and 23132/87), and RPMI-1640 (NCI-N87, TMC-1, TSGH 9201, SK-GT-2, and 23132/87).
MEM (HGC-27) media supplemented with 10% fetal bovine serum, 100 U/mL penicillin G, 100 μg/mL streptomycin sulfate, and 250 ng/mL amphotericin B.

2.2 | Study subjects

Specimens of gastric tissues from 241 consecutive patients who underwent surgical resection of GC at Taipei Medical University Wan Fang Hospital from 1999 to 2011 were retrospectively studied. Of the 241 patients with GC, follow-up information was available for 214 patients (Supplementary Table S1). Tumor and nontumor pairs of gastric tissues were analyzed for CERS6 and SOCS2 expressions. The clinical outcome endpoint was disease-free survival. Retrospective power analysis was conducted to determine the association of CERS6 expression with disease-free survival. This analysis showed that the study had approximately 99% power to detect a significant difference (effect size = 0.54) from the observed data, assuming a significance level of α = 5%. The institutional review board of the hospital approved this study (Approval No. 99049), and written informed consent was obtained from all patients before this study.

2.3 | RNA extraction and quantitative PCR analysis

Total RNA from gastric cells was extracted using TRIzol reagent according to the manufacturer’s protocol (Sigma, St. Louis, MO). After chloroform separation, the aqueous phase was collected, and RNA was precipitated by adding ice-cold isopropanol. After washing once with 75% ethanol, the pellets were air-dried to determine the RNA concentrations. cDNA was synthesized from total RNA using a QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA) following the instructions of the manufacturer. Total RNA was mixed with Genomic DNA Wipeout Buffer and incubated at 42°C for 2 min. Quantiscript RT Buffer, RT Primer Mix, and Quantiscript Reverse Transcriptase were added and incubated at 42°C for an additional 30 min. Primers for CERS6 and GAPDH, which was used as an internal control, and quantitative PCR master mix were purchased from Sigma. The expression levels of the target genes were measured using quantitative PCR in an Illumina Eco Quantitative PCR System (Illumina, Waltham, MA) were used to visualize the targeted proteins. Image processing was performed using ImageMaster software version 5.0 (Amersham Biosciences, Amersham, UK). Experiments were conducted three times independently.

2.5 | Protein extraction and immunoblotting

Total cell and tissue lysates were extracted with RIPA Buffer (Thermo Scientific, Rockford, IL). Denatured protein samples were subjected to 10% SDS-PAGE and transferred to nitrocellulose membranes. Blocked blots were incubated at 4°C overnight with primary antibodies. β-Actin was used as an internal control for equal protein loading. After incubation with secondary antibodies conjugated with peroxidase (Sigma), enhanced chemiluminescence reagents (Perkin Elmer, Waltham, MA) were used to visualize the targeted proteins. Image processing was performed using ImageMaster software version 5.0 (Amersham Biosciences, Amersham, UK). Experiments were conducted three times independently.

2.6 | Immunohistochemistry

Paraffin-embedded sections were stained with primary antibodies at 4°C overnight. A standard peroxidase-conjugated streptavidin-biotin method was used to detect the immunoreactivity (Dako REAL EnVision Detection System; Dako, Carpinteria, CA). Breast cancer tissue was used as a positive control for CERS6. The negative control consisted of the omission of the primary antibody and incubation with 1× PBS (Corning, Manassas, VA). Images were acquired with BX51 microscope (Olympus, Tokyo, Japan). CERS6 or SOCS2 immunoreactivity was assessed semiquantitatively and scored as follows: 0, no staining; 1, weak and focal staining in <25% of the tissue; 2, moderate staining in 25-50% of the tissue; and 3, strong staining in >50% of the tissue. Sections with a score of 0 or 1 exhibited low expression of CERS6 or SOCS2, and those with a score of 2 or 3 were defined as exhibiting high expression or overexpression of CERS6 or SOCS2. Clinical data collection and immunohistochemical analysis were independently performed in an investigator-blinded study.

2.7 | shRNA treatment

For shRNA treatment, SK-GT-2 and 23132/87 cells were infected with lentiviral vectors (two CERS6-shRNA constructs, clone IDs: TRCN00000128836, TRCN0000344342, and one control, clone ID: pLKO.TRCC025, purchased from the National RNAi Core Facility, Taipei, Taiwan) and stable clones resistant to puromycin (Santa Cruz) were selected. Immunoblotting was performed to evaluate the effects of shRNA treatment.

2.8 | Transfection

AGS cells were transfected with Human CERS6 cDNA ORF and empty vectors (OriGene, Rockville, MD) and stable clones resistant to G418 (Dallas, TX). The antibodies against cyclin A, cyclin D, JAK2, p-JAK2, STAT3, p-STAT3, MMP-9, and SOCS2 were purchased from Cell Signaling Technology (Danvers, MA). The antibody against Ki67 was obtained from Novus Biologicals (Littleton, CO). The antibody against β-actin was obtained from Sigma.
2.9 | Colony formation assay

Five hundred cells were seeded into 6-well plates and cultured for 12 days. Individual colonies (>50 cells/colony) were fixed, stained using a solution of 1% crystal violet in methanol, and counted. The plates were scanned with Scanjet 2200c scanner (HP, Palo Alto, CA) and the colony numbers were counted. The percentage of colony formation was calculated by defining the colony number of wild type cells as 100%. The assay was conducted three times, and the results were presented as the mean ± standard deviation (SD).

2.10 | Flow cytometric analysis

The cellular DNA content was determined by flow cytometric analysis of propidium iodide-labeled cells, as described in our previous study.25

2.11 | Wound-healing assay

For the wound-healing assay, cells (5 × 10^5) were seeded into a 12-well culture dish and grown to a nearly confluent monolayer. The monolayers were carefully scratched using a 200 µL pipette tip. Cellular debris was removed by washing with 1× PBS, and the cells were then incubated for 18 h. The cultures were photographed (100× magnification, with Leica DMRB microscope, Leica, Wetzlar, Germany) at 0 and 18 h to monitor the migration of cells to the wounded area, and the number of migrated cells was quantified using Image J software. The percentage of cell migration was calculated by defining the number of migrated wild type cells as 100%. All experiments were performed in triplicate, and the results were presented as the mean ± SD.

2.12 | In vitro invasion assay

The cell invasion capability was examined using a Cell Invasion Assay Kit (Merck Millipore, Darmstadt, Germany), following the manufacturer’s instructions. Complete media were first added to 24-well plates. The cells (2 × 10^5) in serum-free media were added to ECMatrix-layered cell culture inserts (containing 8 µm pore size polycarbonate membranes) and cultured for 24 h. Before staining, the cells on the upper surface were removed. Inserts were then dipped in the Staining Solution to stain invaded cells on the lower surface of the membranes. The cultures were photographed (100× magnification, with Leica DMRB microscope), and the number of invaded cells was counted. The percentage of cell invasion was calculated by defining the number of invaded wild type cells as 100%. The assay was conducted three times independently, and the results were presented as the mean ± SD.

2.13 | Gelatin zymography assay

The activities of matrix metalloproteinase-2 (MMP-2) and MMP-9 in the medium were measured by gelatin zymography protease assays, as previously detailed.26 Image processing was performed using Image-Master software version 5.0.

2.14 | Animals

Five-week-old male athymic nude mice (BALB/c nu/nu) were purchased from the National Laboratory Animal Center, Taiwan. All experiments were conducted in accordance with the guidelines of the Chi Mei Medical Center Animal Ethics Research Board (Approval No. 103121520).

2.15 | Tumor cell inoculation for xenograft proliferation

For CERS6 overexpression, animals were divided into two groups (5 mice/group) for tumor cell inoculation. The CERS6-overexpressed and control AGS cells (5 × 10^6) were mixed in a 200 µL Matrigel (Corning, Manassas, VA) and injected subcutaneously on the right hind flank. Tumor volume was measured every few days after inoculation. The mice were photographed (with CANON 60D camera, CANON, Tokyo, Japan) and sacrificed 5 weeks after inoculation. The tumors were then removed and weighed. For CERS6 knockdown, CERS6 knockdown, and control 23132/87 cells were used for inoculation. The mice were sacrificed 2 weeks after inoculation.

2.16 | Statistical analysis

The difference in CERS6 expression between tumor and nontumor tissues in the same patient was analyzed using a paired t test. The differences in growth, migration, and invasion between CERS6 knockdown and control cells were also analyzed using a Student’s t tests. The clinicopathologic parameters of GC were determined based on the American Joint Committee on Cancer classification. We examined some clinicopathologic parameters, including age, gender, depth of invasion, nodal status, distant metastasis, stage, degree of differentiation, and vascular invasion. The correlation between CERS6 expression and the clinicopathologic parameters was examined using the χ^2 test. Survival analysis was first performed using the Kaplan-Meier method, and statistical significance was then determined using the log-rank test. The Cox proportional hazards model was used for univariate and multivariate analyses to determine the relative prognostic effect of CERS6 and other potential prognostic markers. The assumption of proportionality of hazards functions was assessed using graphical methods. The correlation between CERS6 and SOCS2 expressions was examined using the Spearman’s rank correlation analysis. All data were analyzed using SPSS version 17.0 (SPSS, Inc., Chicago, IL). All statistic tests were 2-sided, and a P value of <0.05 was considered significant.

3 | RESULTS

3.1 | CERS6 was upregulated in GC

To investigate the potential significance of CERS6 in the development and progression of GC, we first examined the expression of CERS6 in
gastric cell lines. Quantitative PCR analysis indicated that CERS6 mRNA was ubiquitously expressed at higher levels in a panel of 7 human GC cell lines than in human normal gastric cell line Hs738.St/Int (Figure 1A). In parallel, as shown in Figure 1B, immunoblotting also revealed that CERS6 protein expression was markedly increased in all 7 GC cell lines as compared with that in Hs738.St/Int cells. An independent cohort comprised of 214 GC patients was enrolled to validate the results from cellular study. Immunohistochemical analysis showed that CERS6 was expressed at higher levels in tumor tissues than in nontumor tissues. Thirty-nine percent of the tumors were totally negative (score 0), and 28% were weak, and focal staining in <25% of tissues (score 1) (Figures 1C and 1D). Thirty-three percent of the tumors revealed high expression or overexpression of CERS6 (21% with a score of 2% and 12% with a score 3, Figure 1E). Furthermore, immunoblotting also shown that CERS6 protein expression was expressed at higher levels in tumor tissues than in nontumor tissues (Supplementary Figure S1). These data strongly suggested that CERS6 expression is significantly elevated in GC.

3.2 | CERS6 upregulation correlated with GC clinicopathologic characteristics and the survival of GC patients

The observed upregulated expression of CERS6 in GC prompted us to further investigate the clinical relevance of CERS6 in the progression of GC. As shown in Table 1, the level of CERS6 expression closely correlated with depth of invasion ($P = 0.0273$), nodal status ($P = 0.0029$), distant metastasis ($P < 0.0001$), stage ($P = 0.0004$), and vascular invasion ($P = 0.0003$).

Furthermore, Kaplan-Meier analysis using the log-rank test showed that inferior disease-free survival was significantly associated with CERS6 overexpression ($P < 0.001$) (Figure 2A). At 5 years, 38 CERS6-low patients were at risk, and the disease-free survival was 0.656 (95% confidence interval [CI] 0.568 to 0.744); 3 CERS6-high patients were at risk, and the disease-free survival was 0.252 (95%CI 0.117 to 0.387).

Inferior overall survival was also significantly associated with CERS6 overexpression ($P < 0.001$) (Figure 2B). At 5 years, 39 CERS6-low patients were at risk, and the disease-free survival was 0.554 (95% CI 0.464 to 0.644); 3 CERS6-high patients were at risk, and the disease-free survival was 0.174 (95%CI 0.041 to 0.307).

Table 2 summarizes the univariate analysis of prognostic biomarkers and patient survival. CERS6 overexpression (Hazard ratio [HR] 3.475, 95%CI 2.260 to 5.344, $P < 0.001$), depth of invasion (HR 8.330, 95%CI 3.621 to 19.164, $P < 0.001$), nodal status (HR 7.915, 95% CI 3.806 to 16.461, $P < 0.001$), distant metastasis (HR 18.711, 95%CI 9.030 to 38.771, $P < 0.001$), and stage (HR 9.161, 95%CI 4.836 to 17.354, $P < 0.001$) were significantly correlated with disease-free survival.

In the multivariate analysis, distant metastasis (HR 8.051, 95%CI 3.571 to 18.154, $P < 0.001$) and stage (HR 3.257, 95%CI 1.138 to 9.327, $P = 0.028$) were prognostically independent. CERS6 overexpression was not prognostically independent but with a borderline association (HR 1.625, 95%CI 0.944 to 2.797, $P = 0.080$) (Table 2).

**FIGURE 1** CERS6 expression in gastric cell lines and tissues. Endogenous CERS6 mRNA (A) and protein (B) expressions were remarkably increased in GC cell lines (AGS, NCI-N87, TMC-1, TSGH 9201, SK-GT-2, HGC-27, and 23132/87). The mRNA levels were measured by quantitative PCR and the protein levels were measured by immunoblotting. Hs738.St/Int cells were the human normal gastric cell line. GAPDH and β-actin were used as internal controls for quantitative PCR and immunoblotting, respectively. The blots in the figure were cropped. C-E, GC analyzed by immunohistochemistry with an antibody against CERS6. Panel C shows a sample without CERS6 expression; Panel D shows a sample with low CERS6 expression; Panel E shows a sample with high CERS6 expression. Magnification: 200×
Collectively, high CERS6 expression seemed to be a risk factor predicting poor survival, suggesting that increased expression of CERS6 likely contributes to GC pathogenesis and might represent a prognostic biomarker for the disease.

### 3.3 The effect of CERS6 overexpression on the prognosis of advanced-stage GC

Tumor stage is an important prognostic biomarker of GC. We therefore determined the effect of CERS6 overexpression on the prognosis of early-stage (stages I and II) and advanced-stage (stages III and IV) GC. The data show that advanced-stage GC concomitant with CERS6 overexpression pointed to a significantly lower 5-year overall survival rate than advanced-stage GC without CERS6 overexpression (Figure 2C, \( P < 0.001 \)), whereas early-stage GC (stages I and II) was associated with a better 5-year overall survival rate regardless of CERS6 expression status (Figure 2D, \( P = 0.068 \)).

### 3.4 Silencing CERS6 suppressed cell proliferation and caused cell-cycle arrest in GC cells

Based on the expression level of CERS6, 2 GC cell lines (SK-GT-2 and 23132/87) with high CERS6 levels were chosen to help us elucidate the role of endogenous CERS6 in the modulation of cell proliferation. The SK-GT-2 and 23132/87 cells were infected with CERS6-shRNA lentiviral vectors to generate CERS6-knockdown SK-GT-2 and 23132/87 cells (Figure 3A). As shown in Figure 3B, the abilities of both cell lines to form colonies were compromised by CERS6 knockdown as compared with corresponding scrambled control cells (quantification data from 23132/87 cells are shown in Supplementary Figure S2A). These results suggest that knockdown of CERS6 suppressed the ability of GC cells to proliferate in vitro.

To dissect the biological events accompanying alterations in cell proliferation caused by CERS6, flow cytometric analysis was applied to analyze changes in DNA content throughout the various phases of the cell cycle. As shown in Figure 3C, CERS6 knockdown 23132/87 cells...
displayed a significant increase in the percentages of cells in G2/M phase. The percentage of CERS6-knockdown cells in G2/M phase was 62.7% and that of scrambled control cells in G2/M phase was 39.9%. Thus, our shRNA experiments suggest that CERS6 knockdown interferes with the G2/M-G1 transition of cell-cycle progression and consequently abrogates the proliferation of GC cells.

3.5 | Overexpressing CERS6 accelerated the proliferation of GC cells

Based on the expression level of CERS6, a GC cell line with a low CERS6 level (AGS) was chosen to help clarify the biological function of CERS6. The AGS cells were transfected with CERS6 cDNA ORF vectors and stable clones were selected (Figure 3A). As shown in Figure 3B, a colony formation assay revealed that after transfection with CERS6 cDNA ORF vector, AGS cells formed more colonies (quantification data are shown in Supplementary Figure S2B). In parallel, ectopic CERS6 expression did not significantly change the percentage of cells in the G2/M phase (Figure 3C). The percentage of CERS6-overexpressing cells in G2/M phase was 34.0% and that of scrambled control cells in G2/M phase was 34.8%. Overall, our overexpression experiments indicate that CERS6 might function as an oncoprotein capable of facilitating the cell cycle and thus accelerating the progression of cell proliferation.

3.6 | Abrogating CERS6 repressed cell cycle regulators cyclins A, B, CDK1, and the JAK2/STAT3 signaling pathway

To identify the molecular mechanisms that govern the CERS6 knockdown-induced G2/M arrest, we assessed the expression of various cyclins involved in cell cycle control in the CERS6 knockdown GC cells. Reduced expressions of cyclins A and B, both of which are involved in the regulation of G2 phase, was observed in the CERS6 knockdown 23132/87 GC cells (Figure 3D). By contrast, the expressions of cyclins D and E, both involved in the regulation of G1 phase, remained unchanged (Figure 3D). CERS6 knockdown also exhibited inhibitory effect on the expression of
By contrast, the expression of CDK2 remained unchanged (Supplementary Figure S3). Previous studies have shown that the enzyme activities of JAK2 and STAT3 are regulated by phosphorylation and that the JAK2/STAT3 signaling pathway is involved in the G2/M cell cycle arrest. We also analyzed the influence of CERS6 knockdown on the phosphorylation of JAK2/STAT3. As shown in Figure 3E, CERS6 knockdown exhibited inhibitory effects on the levels of phospho-JAK2 and phospho-STAT3 but not those of their unphosphorylated forms. Taken together, these data suggest in part that CERS6 knockdown induced G2/M cell cycle arrest by inhibiting the JAK2/STAT3 signaling pathway and, in turn, the expressions of cyclins A and B.

### 3.7 Augmenting CERS6 increased cell cycle regulators cyclins A and B and the JAK2/STAT3 signaling pathway

CERS6-overexpressing AGS cells were used to validate the results from 23132/87 cells. Elevated expressions of cyclins A and B were observed in the CERS6-overexpressing AGS cells (Figure 3D). As the results from 23132/87, the expressions of cyclin D and cyclin E still remained unchanged (Figure 3D). Furthermore, ectopic CERS6 expression exhibited stimulatory effects on the levels of phospho-JAK2 and phospho-STAT3 (Figure 3E). These data also indicate that CERS6 played an important role in cell proliferation.

### 3.8 Overexpression of CERS6 in clinical samples correlated with increased levels of phosphorylated JAK2/STAT3

Immunoblotting was employed to further understanding the correlation between CERS6 expression and the levels of phosphorylated JAK2/STAT3 in clinical samples. As shown in Supplementary Figure S1, overexpression of CERS6 also correlated with increased expression of phosphorylated JAK2/STAT3 in tumor tissues than in nontumor tissues.

### 3.9 Inhibiting CERS6 decreased the migration and invasion of GC cells

To verify the effect of CERS6 knockdown on the migration of GC cells, we performed a wound-healing assay and observed a significant delay in wound closure in CERS6 knockdown SK-GT-2 cells compared with the scrambled control cells (Figure 4A; quantification data are shown in Supplementary Figure S4A). In the cell invasion assay, CERS6 knockdown significantly suppressed cell invasion compared with the scrambled control (Figure 4A, quantification data are shown in Supplementary Figure S4A).

MMP-2 and MMP-9 are involved in the breakdown of extracellular matrix in disease processes such as, for example, metastasis. They can be inhibited by specific endogenous tissue inhibitors of metalloproteinases (TIMPs), including TIMP-1 and TIMP-2. Elevated expression level of uPA is found to be correlated with tumor malignancy. It is believed that the tissue degradation following plasminogen activation facilitates tissue invasion and thus contributes to metastasis. We used immunoblotting to analyze the effects of CERS6 knockdown on the expression of the above-mentioned metastasis-related molecules. As shown in Figure 4C, CERS6 knockdown decreased the expressions of MMP-2, MMP-9, uPA, and uPAR. Upregulated expressions of the MMP inhibitors TIMP-1 and TIMP-2 were found after CERS6 knockdown. In addition, the gelatin zymography assay showed that CERS6 knockdown significantly inhibited the activities of MMP-2 and MMP-9 compared with the scrambled control (Figure 4B). These results suggest that CERS6 knockdown suppresses cell spreading by dysregulating the expression of metastasis-related molecules.

### 3.10 Forced expression of CERS6 increased the migration and invasion of GC cells

CERS6-overexpressing AGS cells were used to confirm the results from 23132/87 cells. Supporting the role of CERS6 in promoting cell motility, CERS6 overexpression in AGS cells

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<th>Variable</th>
<th>Univariate</th>
<th>Multivariate</th>
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<tr>
<td></td>
<td>HR (95%CI)</td>
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<td>CERS6</td>
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<td>Low expression vs high expression</td>
<td>3.475 (2.260-5.344)</td>
<td>&lt;0.001</td>
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<td>Depth of invasion</td>
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<td>T1 + T2 vs T3 + T4</td>
<td>8.330 (3.621-19.164)</td>
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<td>Nodal status</td>
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<td>N0 vs. N1 + N2 + N3</td>
<td>7.915 (3.806-16.461)</td>
<td>&lt;0.001</td>
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<td>Distant metastasis</td>
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<td>Absence vs presence</td>
<td>18.711 (9.030-38.771)</td>
<td>&lt;0.001</td>
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<td>I + II vs III + IV</td>
<td>9.161 (4.836-17.354)</td>
<td>&lt;0.001</td>
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*All statistical tests were 2-sided. Significance level: P < 0.05.
increased migration and invasion (Figure 4A; quantification data are shown in Supplementary Figure S4B). Furthermore, CERS6 overexpression upregulated the expressions of MMP-2, MMP-9, uPA, and uPAR, and inhibited the expressions of TIMP-1 and TIMP-2 (Figure 4C). Consistent with MMP-2 and MMP-9 immunoblotting, the gelatin zymography assay showed that ectopic CERS6 expression significantly increased the activities of MMP-2 and MMP-9 (Figure 4B).
3.11 | CERS6 promoted GC tumor growth in vivo

The in vitro data that CERS6 positively modulated the GC cell cycle and proliferation prompted us to ask whether CERS6 acted as an endogenous oncoprotein in vivo. In this study, each experimental mouse bearing CERS6 knockdown or scrambled control 23132/87 cells on the right hind flank began to exhibit conspicuous difference in tumor growth between these two groups within the first week, and the difference continued to expand through the experimental endpoint, at which time the tumors of the control group displayed twofold higher weight than the CERS6 knockdown tumors (Figure 5B). In parallel, this trend was also confirmed by the sizes of dissected tumors (Figure 5C). Figure 5D revealed that CERS6 knockdown significantly decreased the mitotic index, shown as the expression of Ki67, compared with the control group. This result suggested that the decreased tumor volume was due to the decelerated proliferation. All animals appeared healthy, with no loss of body weight noted during experiments (Supplementary Figure S5A).

On the other hand, mice bearing CERS6-overexpressing or vector control AGS cells were used to validate the results from 23132/87 cells. The CERS6-overexpressing tumors showed 2.6-fold higher weight than the vector control tumors (Figure 5A). The sizes of dissected tumors also confirmed this trend (Figure 5C). Figure 5D showed that CERS6 overexpression significantly increased the mitotic index compared with the control group. All animals appeared healthy, with no loss of body weight noted during experiments (Supplementary Figure S5B). Collectively, these data strongly suggest that CERS6 markedly accelerated the proliferation of tumor cells.

3.12 | CERS6 exerted its oncogenic function by suppressing SOCS2

In order to further elucidate the mechanism of CERS6-involved tumorigenesis, after literature survey, suppressor of cytokine signaling 2 (SOCS2) was chosen for further investigation of its involvement in signal transduction. SOCS proteins were originally described as the feedback inhibitors of JAK/STAT signaling.29,30 The downregulation of SOCS2 has already been demonstrated in several cancers.31,32 To the best of our knowledge, the expression of SOCS2 in GC remains unknown. Next, we assessed the expression of SOCS2 in 214 gastric tissues. Immunohistochemical analysis showed that SOCS2 was expressed at lower levels in tumor tissues than in paired nontumor
tissues (Figures 6A and 6B). As shown in Supplementary Table S2, in GC tissues, the expression level of CERS6 closely correlated with that of SOCS2 ($P < 0.0001$). Statistically significant inverse correlation was observed between CERS6 levels and expression of SOCS2 (Supplementary Table S3, $r = -0.702$, $P < 0.001$, Spearman’s rank correlation analysis). In addition, as shown in Figure 6C, immunoblotting revealed that SOCS2 protein expression was markedly decreased in AGS and 23132/87 GC cells as compared with that in Hs738.St/Int cells. By comparing Figures 1B and 6C, we found a correlation between CERS6 overexpression and the downregulation of SOCS2. This correlation was further confirmed by CERS6 manipulation. Stable CERS6 overexpression and knockdown, respectively, significantly decreased and increased the expression of SOCS2 (Figure 6C). We therefore conclude that CERS6 was involved in the expression of SOCS2.

**FIGURE 5** Effect of CERS6 manipulation in AGS and 23132/87 cells on in vivo tumor growth in nude mice. A, Stable CERS6 overexpression resulted in the significant enhancement of AGS xenograft proliferation. The histogram represents the results of the average tumor weight (presented as the mean ± SD). vec: control vector-transfected GC cells; CERS6: CERS6-overexpressing GC cells. B, Stable CERS6 knockdown resulted in the significant suppression of 23132/87 xenograft proliferation. The histogram represents the results of the average tumor weight (presented as the mean ± SD). con: scrambled control GC cells; shCERS6: CERS6-knockdown cells. C, Stable CERS6 overexpression and knockdown resulted in time-dependent increase and decrease of the volume of xenograft. The results are presented as the mean ± SD ($n = 5$). D, Stable CERS6 overexpression and knockdown resulted in a significant increase and decrease of Ki67 expression in xenografts, examined by immunohistochemistry. Magnification: 200×.
Figure 7 summarizes a model of how CERS6 regulates the JAK2/STAT3 pathway and promotes proliferative and metastatic signals in GC cells. In normal cells, JAK2/STAT3 pathway was blocked through dephosphorylation by endogenous SOCS2. Then the expressions of cell cycle-control and metastasis-related gene decreased, which restrained GC cell growth, spread, and xenograft proliferation. On the contrary, in cancer cells, overexpressed CERS6 downregulates SOCS2, which in turn, leads to an activation of the JAK2/STAT3 pathway by phosphorylation and increased cell cycle-control and metastasis-related gene expression resulting in GC cell growth, spread, and xenograft proliferation.

Figure 6 SOCS2 protein expression in gastric tissues and cells. A and B, Gastric tissues analyzed by immunohistochemistry with an antibody against SOCS2. Panel A shows a nontumor sample with high SOCS2 expression; Panel B shows a tumor sample with low SOCS2 expression. Magnification: 200×. C, SOCS2 protein expression was markedly decreased in AGS and 23132/B7 GC cells as compared with that in Hs738.St/Int cells. Stable CERS6 overexpression and knockdown, respectively, significantly decreased and increased the expression of SOCS2. β-actin was used as internal control. The blots in the figure were cropped. vec: control vector-transfected GC cells; CERS6: CERS6-overexpressing GC cells; con: scrambled control GC cells; shCERS6: CERS6-knockdown cells.
pathway by phosphorylation and increased cell cycle-control and metastasis-related gene expression resulting in GC cell growth, spread, and xenograft proliferation.

4 | DISCUSSION

GC remains a major public health problem worldwide: globally, the incidence of GC ranks fourth among all cancer types.\(^1\) Despite improvements in GC treatment methods, the survival rates of patients with advanced GC remain low, and the mortality of GC remains the third highest among all cancers worldwide.\(^1\) Clinically, patients with advanced GC often experience distant metastasis; thus, unlike patients with early GC, patients with advanced GC cannot undergo a resection operation and typically have poor prognosis.\(^{33,34}\) Numerous studies have indicated that GC occurs because of molecular and genetic changes in gastric epithelial cells.\(^{35}\) An improved understanding of the molecular mechanism of GC carcinogenesis can, we believe, help to identify new prognostic biomarkers and to develop new methods for treating this deadly malignant neoplasm.

Previous studies have shown that CERS6 is overexpressed in breast, head and neck, and lung cancers.\(^{18,20,24}\) The present study explored the expression of CERS6 in gastric tissues. The results of immunohistochemistry and immunoblotting experiment have revealed that, as in other types of cancer, CERS6 was overexpressed in both GC tissues and cells. However, the mechanism of CERS6 overexpression remains unclear. Only 1 study found CERS6 to be overexpressed in lung cancer tissues and to be a direct target gene of microRNA-101. The elevated expression of CERS6 has been found to be due in part to the reduced expression of microRNA-101 in lung cancer tissues.\(^{24}\) However, the mechanism for the overexpression of CERS6 in GC remains to be investigated.

The results from the \(\chi^2\) test showed that the expression of CERS6 was correlated with depth of invasion, nodal status, distant metastasis, and stage. These results accord with those of previous studies. Suzuki et al indicated that when the expression of CERS6 in cells was knocked down, the cells' ability to migrate and invade would be impaired. Conversely, when the amount of CERS6 in cells increased, the cells' ability to migrate and invade would be enhanced.\(^{24}\) The authors believe that an increasing amount of CERS6 could increase the amount of C16-ceramide, and that C16-ceramide is related to the formation of lamellipodia. The latter are related to cell migration and invasion, which helps to explain why the expression of CERS6 is correlated with cell migration and invasion. Schifffmann et al conducted a study on breast cancer and found C16-ceramide to be significantly correlated with nodal status, implying that CERS6 is related to nodal status.\(^{19}\) In the present study, we found the expression of CERS6 in GC cells to be positively correlated with the amounts of MMP-9 and MMP-2. When the expression of CERS6 was knocked down, the amounts of MMP-9 and MMP-2 decreased; conversely, when the amount of CERS6 increased, the amounts of MMP-9 and MMP-2 increased as well. Given that MMPs are related to tumor metastasis, we believe that the expression of CERS6 in GC cells facilitates the expression of MMP-9 and MMP-2, resulting in cell migration and invasion. This explains why the overexpression of CERS6 is correlated with clinicopathologic parameters. Regarding the way in which CERS6 regulates the expression of MMPs, Sen et al found that Src oncoprotein could inhibit the expression of SOCS2, and SOCS2 can cause cell death by inhibiting the activities of JAK2 and STAT3. Accordingly, Src facilitates cell survival by inhibiting the expression of SOCS2.\(^{26}\) Hu et al have indicated that the JAK2/STAT3 signaling pathway can regulate the expression of MMPs, and that curcumin can reduce the expression of MMPs by inhibiting the JAK2/STAT3 signaling pathway and thus inhibiting cell migration.\(^{27}\) In the present study, we found that the overexpression of CERS6 in GC cells could inhibit the expression of SOCS2; accordingly, the JAK2/STAT3 signaling pathway would not be inhibited and the amounts of MMP-9 and MMP-2 would be increased. Conversely, when the expression of CERS6 was knocked down, the expression of SOCS2 would not be inhibited; accordingly, the JAK2/STAT3 signaling pathway would be inhibited, thus reducing the amounts of MMP-2 and MMP-9. Thus far, the present study is the first to explore the SOCS2/JAK2/STAT3/MMP-9 and MMP-2 signaling pathway.

Prognosis is critical for patients with GC; the major value of prognostic biomarkers is that they aid in developing an adequate postoperative treatment protocol for patients with GC. Currently, the most crucial prognostic biomarker for GC is tumor stage, as proposed by the American Joint Committee on Cancer. However, the prognosis for GC patients at the same stage often varies; therefore, it is important to identify a prognostic biomarker that is more accurate than tumor stage. Few studies have been conducted on the relationship between ceramide synthases and prognosis. According to Wang et al, the expression of CERS2 is related to the development and progression of bladder cancer in humans; therefore, CERS2 could be a prognostic biomarker for bladder cancer.\(^{29}\) Regarding the relationship between CERS6 and cancer prognosis, only Suzuki et al have found early this year that CERS6 was overexpressed in the tissues and cells of lung cancer and that CERS6 could be a prognostic biomarker for lung cancer.\(^{24}\) In the present study, we found that the overexpression of CERS6 was correlated with poor disease-free survival of patients with GC and that CERS6 could be a prognostic biomarker for GC. To our knowledge, this study is the first to point to this possibility. In addition, the current results revealed that in patients with advanced GC, CERS6 is correlated with poor disease-free survival. Therefore, intensive follow-up programs are needed for patients with advanced GC.

One limitation of this study was the small sample size of the GC cohort. Accordingly, additional cohort studies would be helpful to confirm our findings.

Various types of ceramide synthases can synthesize ceramides of various chain lengths, and ceramides of specific chain lengths may facilitate cell survival during cancer pathogenesis. For example, C16-ceramide produced from CERS6 was found to be related to cancer cell growth; however, the way in which CERS6 facilitates cancer cell growth remains unknown.\(^{18}\) In the present study, we explored the role of CERS6 in GC cell proliferation by manipulating the expression of CERS6, and the results of this study accord with those of previous studies. The overexpression of CERS6 increased the number of colonies, whereas the knockdown of CERS6 reduced them, indicating that CERS6 facilitated cell proliferation. In addition, the knockdown of
CERS6 reduced the number of colonies because the cell cycle is arrested in the G2/M phase (i.e., cell proliferation is slowed). We also obtained consistent results in detecting the expression of cell cycle-related molecules. We found that the amounts of cyclins A and B involved in the G2/M phase were positively correlated with the amount of CERS6; in addition, the amounts of cyclins D and E involved in the G1/S phase remained unchanged. Regarding how CERS6 regulates the expression of cyclins A and B, Reiterer et al found that the JAK2/STAT3 signaling pathway was related to the regulation of the cell cycle: when the signaling pathway was inhibited, the cell cycle would arrest in the G2/M phase. Su has indicated that when Tanshinone IIA was used to treat GC cells, it reduced the expression of cyclins A and B and caused the cell cycle to arrest in the G2/M phase. In the present study, we found that when the expression of CERS6 was knocked down, the expression of SOCS2 was no longer inhibited; accordingly, the JAK2/STAT3 signaling pathway was inhibited and the expression of cyclins A and B was reduced, causing the cell cycle to arrest in the G2/M phase. Thus far, this study is the first to explore the SOCS2/JAK2/STAT3/cyclins A and B signaling pathway.

A better understanding of the etiology of GC can facilitate the development of new drugs with which to treat GC. According to our study, CERS6 is overexpressed in GC tissues and cells; knockdown the expression of CERS6 can inhibit cell proliferation and spread as well as tumor growth in vivo. Previous studies have indicated that fumonisin B1 is an inhibitor of CERS6. In the near future, we intend to explore whether fumonisin B1 has the ability to inhibit GC cell growth.

In summary, we found that overexpression of CERS6 in GC cells facilitated cell proliferation and spread as well as xenograft proliferation, resulting in a significantly worse prognosis of the patients with GC. Our findings in this study suggest that CERS6 overexpression can be a useful biomarker for predicting the outcomes of GC patients and that CERS6 targeting represents a potential modality for treating GC.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of the article.