Original Paper

CLIC1 Promotes the Progression of Gastric Cancer by Regulating the MAPK/AKT Pathways

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Key Words

Chloride intracellular channel 1 • Gastric cancer • Integrins • MAPKs • AKT

Abstract

Background/Aims: Chloride intracellular channel 1 (*CLIC1*), which is a member of the chloride channel protein family, is associated with various human tumors. Recent studies have shown that *CLIC1* is involved in the occurrence and development of gastric cancer (GC). However, the exact mechanism remains unclear in GC. **Methods:** Effects of *CLIC1* on the progression of GC *in vivo* and *in vitro* and the potential underlying mechanisms have been investigated by analysing 54 patients with GC, as well as human gastric cell lines SGC-7901 and MGC-803, utilizing proteomics, RT-PCR, Western blotting, flow cytometry, Cell invasion and migration assays and xenograft tumor models. **Results:** Our study shows that *CLIC1* knockdown by targeted-siRNA markedly inhibits GC cell invasion and migration and induces apoptosis *in vitro*. In total, 54 differentially expressed proteins were identified in GC cells SGC-7901 after *CLIC1* silencing by isobaric tags for relative isotope labeled and absolute quantitation (iTRAQ) technology, including integrin $\alpha 1$ (ITG $\alpha 1$) and ITG $\alpha 3$. The expression levels of ITG $\alpha 3$, ITG αv , ITG $\beta 1$ and Bcl-2 mRNA and protein were decreased significantly in GC cells after *CLIC1* knockdown; ITG $\alpha 1$ and Fas were upregulated, but the level of survivin was not significantly different. GC growth and metabolism were decreased *in vivo* after *CLIC1* silencing, but apoptosis was markedly increased. Further study showed that the expression levels of ITG $\alpha 3$, ITG αv and ITG $\beta 1$, as well as AKT-phosphorylation, ERK-phosphorylation and p38-phosphorylation, were reduced *in vivo* after *CLIC1* knockdown, while ITG $\alpha 1$ was upregulated. **Conclusions:** We speculate that *CLIC1* may play an important role in the progression of GC, and its mechanism may be related to the regulation of integrin family proteins, which leads to the sequential regulation of the PI3K/AKT, MAPK/ERK and MAPK/p38 pathways.

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Introduction

Gastric cancer (GC) is a gastrointestinal malignancy originating from the epithelium and is one of the most common malignant tumor types. An estimated 951,600 new GC cases and 723,100 deaths occurred worldwide in 2012. Recent studies have shown that GC was the second leading cause of cancer death worldwide, accounting for 6.8% of cancer incidence and 8.8% of cancer deaths [1]. To date, multimodal treatment strategies, including chemotherapy, radiotherapy, and surgery, have limited the progression of tumors remarkably, and the rate of systemic metastasis has been substantially reduced. However, tumor metastasis and recurrence are the main causes of death of GC patients. Additionally, the mechanisms underlying the invasion and metastasis of GC remain to be elucidated.

Chloride intracellular channel 1 (CLIC1) was the first protein identified from the CLIC family, and is the most studied family member. *CLIC1* is located on human chromosome 6p21.3, with a total length of 1,223 basepairs that encode a 241-amino acid ion channel protein. CLIC1 may play an important role both in the normal physiological cell state and the development of tumors because it is expressed in the cell nucleus, cytoplasm and cell membrane [2]. CLIC1 may be involved in the physiological cell state, such as changes in cell volume, the regulation of membrane potential, the acidification of intracellular organelles, the proliferation and differentiation of cells and cell cycle progression. However, the abnormal expression of CLIC1 can lead to abnormal physiological regulation and may affect the occurrence and development of tumors, especially in terms of invasion and metastasis [3].

Early studies have demonstrated the role of CLIC1 in invasion and metastasis in several malignant tumor types, such as liver cancer, colorectal cancer and prostate cancer, indicating that it may play an important role in the progression of cancer [4-6]. Recently, Chen et al. [7] reported that the CLIC1 protein was over-expressed in human GC tissues, and its expression was correlated with lymph node metastasis, lymphatic vessel and nerve infiltration, pathological stage and survival rate. Zheng et al. [8] confirmed that the over-expression of *CLIC1* promoted the invasion and metastasis of GC cells. Additionally, knockdown of *CLIC1* via transient transfection was shown to suppress invasion and metastasis of GC cells. Since the mechanisms involving signaling pathways regulated by CLIC1 remain unclear in GC, we designed this study to investigate the effect of *CLIC1* silencing on apoptosis, migration and invasion of GC *in vitro* and *in vivo*, aiming to explore potential molecular signaling pathways.

Materials and Methods

Ethics approval

This study was approved by the Ethical Review Committee of the First Affiliated Hospital of Guangxi Medical University. Written informed consent was obtained from all patients.

Study population

In total, 54 patients (38 males and 16 females) with GC diagnosed between 2013 and 2015 were enrolled in this study. Each patient had undergone gastric resection (22 total gastrectomies and 32 partial gastrectomies). The study protocol was approved by the Medical Ethics and Human Clinical Trial Committee at the First Affiliated Hospital of Guangxi Medical University. We collected the following data: patient age and gender; tumor size; lymph node metastasis; distant metastasis; pathological type; and TNM stage. Pathological type features were classified as two types: (i) poorly differentiated adenocarcinoma; and (ii) undifferentiated, mid-differentiated or well differentiated.

Cell culture

The human gastric cell lines SGC-7901 and MGC-803 were procured from the cell bank of the Chinese Academy of Science (Shanghai, China). All cells were cultured in RPMI-1640 medium (HyClone, Logan, UT, USA) supplemented with 10 % fetal bovine serum (HyClone, Logan, UT, USA) at 37°C, 5 % CO₂ in a standard humidified incubator. Cells in the logarithmic phase were used for the experiments.

Lentivirus-mediated RNA interference (RNAi)

For lentivirus-mediated silencing of *CLIC1* (NM_001288.4), one effective sequence of human *CLIC1*siRNAs (small interference RNAs) was selected, as follows: 5'-GCCAAAGUUACACAUAGUATT-3'. Another fragment with the scrambled sequence (5'-TTCTCCGAACGTGTCACGT-3') had no significant homology to human and mouse gene sequences and was chosen as a negative control siRNA. These siRNAs were synthesized and inserted into the GV115 lentivirus vector. The positive clones were verified by DNA sequencing. Then, the recombinant lentivirus was packaged and concentrated, and the final virus titer was determined. SGC-7901 and MGC-803 cells were infected with the recombinant lentivirus at a multiplicity of infection of 40 and 10, respectively, for 16h. The medium was then replaced with fresh medium. The cells were observed under fluorescence microscopy after 3 days.

qRT-PCR

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The concentration and purity of the RNA samples were detected at 260 nm. First-strand cDNA was generated through reverse transcription with reverse transcriptase (Promega, Madison, WI, USA). The specific primers were synthesized by Genechem (Shanghai, China) as follows: *GAPDH*, 5'-TGACTTCAACAGCGACACCA-3' (forward) and 5'-CACCTGTTGCTGTAGCCAAA-3' (reverse); *CLIC1*, 5'-ACCGCAGGTCGAATTGTTC-3' (forward) and 5'-ACGGTGGTAACATTGAAGGTG-3' (reverse); *ITGa1*, 5'-GTGATAGGAGCTCCGCTGGAA-3' (forward) and 5'-ACATCTGTCAGACCGTCACCATTTA-3' (reverse); *ITGa3*, 5'-ATTATTGAGGACATGTGGCTTGGAG-3' (forward) and 5'-TGACCACAGCACCTGGGTGTA-3' (reverse); *ITGav*, 5'-AAACTCGCCAGGTGGTATGTGA-3' (forward) and 5'-CTGGTGCACACTGAAACGAAGA-3' (reverse); *ITGβ1*, 5'-AGCTGAAGACTATCCATTGACCTC-3' (forward) and 5'-TGGTGTGTGCTAATGTAAGGCATC-3' (reverse). SYBR Premix Ex Taq (TaKaRa, Dalian, China) was used for real-time PCR, and the total reaction volume was 20 μl. The cycling conditions included a holding step at 95°C for 30s, and 45 cycles done in two steps: 5 s at 95°C and 30 s at 60°C, and a dissociation step was added to verify that each primer pair produced only a single product at 15 s at 95°C, 30 s at 55°C and 15 s at 95°C. *GAPDH* was used as the internal control, and gene expression was quantified using the 2^{-ΔΔCt} method.

iTRAQ (isobaric tag for relative and absolute quantitation) screening of differential proteomics

The cells were harvested for the analysis of protein expression and lysed on ice using RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China). The protein concentration of the lysate was determined by the bicinchoninic acid assay (Beyotime Biotechnology, Shanghai, China). The total protein was digested with Trypsin (Beyotime Biotechnology, Shanghai, China) with the ratio of protein: trypsin = 40:1 at 37°C for 16 h. After trypsin digestion, the peptides were dried by vacuum centrifugation. iTRAQ labeling was carried out based on the manufacturer's instructions and each experiment was performed in two biological replicates. The peptides were labeled with the iTRAQ Reagents Multiplex kit (Applied Biosystems, Foster City, CA, USA) and were reconstituted in 70 μL ethanol. The control sample and *CLIC1*siRNA sample were labeled with iTRAQ reagents 114 and 115, respectively. All tagged samples were incubated for 1 h at room temperature and were then pooled together and lyophilized to powder. The labeled samples were purified using a strong cation exchange chromatography (SCX) column (Phenomenex Inc., Torrance, CA, USA) and separated by liquid chromatography (LC) using the LC-20AB HPLC Pump system. The iTRAQ-labeled peptide mixtures were dissolved in 200 μL buffer A [10 mM NaH₂PO₄ in 20% ACN (acetonitrile), pH 3.0] and loaded onto a 100 × 2.1 mm Ultremex SCX column (Phenomenex) containing 5-μm particles. The mobile phase consisted of buffer A, and buffer B (10 mM KH₂PO₄, 500 mM KCl, and 20% ACN, pH 3.0) was set at a flow rate of 0.25 ml/min. An optimized gradient elution mode was carried out as follows: 0–15 min, 0%B; 15–50 min, 0%–40%B; 50–55 min, 40%–100%B; 55–63 min, 100%B; 63–65 min, 100%–0% B. Elution was monitored by measuring the absorbance at 214 nm, and fractions were collected every 1 min. The eluted peptides were pooled into 20 fractions, desalted with a Strata X C18 column (Phenomenex Inc., Torrance, CA, USA) and vacuum-dried [9]. The nanoscale HPLC system (EASY-nLC from Proxeon Biosystems) connected to an Orbitrap Q Exactive equipped with a nanoelectrospray source was used to separate the peptides, and then the peptide samples were loaded onto the EASY C18 column (Thermo Scientific) after reconstituting in mobile phase A (2% ACN and 0.1% formic acid). The liquid phase condition was performed as follows: flow rate of 300 nl/min; 0–5 min, 0%B (98% ACN and 0.1% formic acid); 5–50 min, 5%–35%B; 50–52 min, 35%–100%B; 52–55 min, 100%B. The effluent from HPLC was directly electrosprayed into the mass spectrometer for analysis [10], then the SEQUEST Cluster software was used to search the Uniprot (<http://www.uniprot.org/>), IPI (<http://www.uniprot.org/>).

www.ebi.ac.uk/IPI/) and NCBI (<https://www.ncbi.nlm.nih.gov/>) databases for differentially expressed proteins.

Western blotting

The protein extraction and concentration determination were similar to the above-mentioned method. Forty micrograms of protein sample per well were resolved by SDS-PAGE and transferred to a PVDF membrane and blocked with 5% BSA (bull serum albumin) in TBST (Tris buffered saline and Tween 20). The membrane was incubated with primary antibodies against Bcl-2 (Cell Signaling Technology, Danvers, MA, USA), survivin (Cell Signaling Technology), Fas (Cell Signaling Technology), ITGαv (Abcam, Cambridge, MA, UK), ITGα1 (Abcam), ITGα3 (Abcam), ITGβ1 (Abcam) and β-actin (Cell Signaling Technology) and were then incubated with HRP-conjugated secondary antibody (Cell Signaling Technology). Immunoreactivity signals were developed using the ECL reagent (Beyotime Biotechnology) and recorded by a fluorescence detection system.

Cell apoptosis analysis

An annexin V-PE Apoptosis Detection Kit (Becton Dickinson, San Diego, CA, USA) was used to detect cell apoptosis following the manufacturer's instructions. In brief, the cells were collected by centrifugation at 2000 rpm for 5 min. After washing, the cells were resuspended in 100 μl of annexin V binding buffer, then 5 μl annexin V-PE and 5 μl 7-Amino-actinomycin D were added to the solution. After incubation in the dark for 15 min at room temperature, 400 μl of binding buffer was added, and the cells were subjected to flow cytometry (FCM).

Cell invasion and migration assay

The invasive capacity of the cells after transfection was measured using a Transwell chamber assay. Matrigel (Becton Dickinson) was put into a 4°C refrigerator overnight to melt. The next day, the Matrigel concentration was diluted by 1.25 mg/ml with serum-free medium, and 50 μl Matrigel was added to the upper chamber of each Transwell insert (pore size 8 μm, Corning, NY, USA). Next, they were incubated at 37°C for 5 h to convert the Matrigel from liquid to gelatin. After hydration of the Matrigel with serum-free medium, 100 μl SGC-7901 and MGC-803 cells with a density of 3×10^5 /ml and 2×10^5 /ml, respectively, were placed in the upper chamber of each Transwell insert, and 500 μl of RPMI 1640 supplemented with 10% fetal bovine serum was added to the lower compartment of the cell culture insert. After a 24-h incubation at 37°C in 5% CO₂, the cells on the upper surface of the Transwell insert were completely removed by wiping with a cotton swab. The invaded cells that remained on the lower surface of the filter were fixed and stained with 1% crystal violet (Sigma-Aldrich, USA) solution and 10% ethanol. The stained cells in five randomly selected microscopic fields per filter were counted. A cell migration assay was also performed using a Transwell chamber assay (pore size 8 μm, Corning, NY, USA) without Matrigel. Other treatments were the same as the cell invasion assay.

Orthotopic xenografted tumor models

The animal experiments were approved by the Animal Ethics Committee of The First Affiliated Hospital of Guangxi Medical University. Female BALB/C nude mice (5–6 weeks old, weighing about 18–20 g) were housed in an animal facility with 50%–65% relative humidity, 22°C–24°C environmental temperature and a 12-h light/dark cycle. The BALB/C nude mice were randomly divided into three groups (n = 6/group). Then, CLIC1 knockdown or siRNA control SGC-7901 and MGC-803 cells (6×10^7 in 200 μl medium) were injected subcutaneously into the armpit area. However, the MGC-803-KD xenografted tumor model failed even when the experimental conditions were optimized. Through a literature search, we found that an siRNA with a special chemical modification (chol-siRNA) can be directly injected into specific tissues or organs to silence a target gene [11]. Thus, both CLIC1-chol-siRNA and MGC-803 cells were injected subcutaneously into the armpit area, and MGC-803-chol subcutaneous tumors were successfully established. The subcutaneous tumors were sliced and inoculated in the armpit area again. Then, the subcutaneous tumors of the second generation were sliced for constructing orthotopically transplanted tumors. The tumor volume was calculated by $0.5 \times \text{length} \times \text{width}^2$. Protein Simple Wes (WES) technology was used to detect the protein expression from the tumor tissues. The validation of transplanted tumor tissues was tested by hematoxylin-eosin (HE) staining, and cell apoptosis of the tumor tissues was detected using the terminal deoxynucleotidyl

transferase-mediated dUTP nick end-labeling (TUNEL) Assay by 3, 3'-Diaminobenzidine (DAB) and fluorescence staining. The lymph node metastasis and metabolic status of the orthotopically transplanted tumors were evaluated using PET-CT. The protein content of the tumors was detected using WES assays [12, 13], and the primary antibodies were as follows: CLIC1(Abcam), GAPDH(Cell Signaling Technology), Bcl-2(Cell Signaling Technology), Bax(Abcam), ITG α v(Abcam), ITG α 1(Abcam), ITG α 3(Abcam), ITG β 1(Abcam), ERK(Cell Signaling Technology), p-ERK(Cell Signaling Technology), AKT(Abcam), p-AKT (Abcam), p38(Cell Signaling Technology) and p-p38(Cell Signaling Technology).

Statistical analysis

All experiments were performed at least in triplicate. The data is expressed as mean $\pm$ SD (standard deviation). Differences among the means of multiple groups were compared using analysis of variance (ANOVA). The t test was used to compare the difference between two groups. Statistical significance was determined at $P < 0.05$.

Results

Association of CLIC1 expression with clinicopathological characteristics

Representative staining results of CLIC1 are presented in Fig. 1A-B, which show the expression of the protein in the cytoplasm and nucleus. The results show that CLIC1 staining in GC was higher than that of adjacent noncancerous tissue ($P < 0.001$) (Fig. 1C). Clinicopathological characteristics including patient sex, age, whether lymph node metastasis occurred, whether distant metastasis occurred, pathological type and TNM stage of the patients enrolled in this study are listed in Table 1. As shown, a higher CLIC1 positive rate was observed in patients with distant metastasis than in those without metastasis ($P < 0.001$). Moreover, higher CLIC1 expression was observed in patients with the worst TNM stage ($P = 0.028$).

GC cell lines with low CLIC1 expression were constructed

The CLIC1-siRNA sequence was successfully inserted into the GV115 lentivirus vector and the positive clones were verified by DNA sequencing (Fig. 2A). The cell transfection was performed by lentivirus-mediated RNAi. The transfection efficiency was evaluated under a fluorescence microscope. Our results showed that the transfection efficiency was over 80% in both SGC-7901 and MGC-803 cells (Fig. 2B-C). The CLIC1 mRNA expression in SGC-7901 and MGC-803 cells was significantly lower than that in the negative control (NC) and blank control (CON) groups after lentivirus-mediated RNAi ($P < 0.05$). The inhibition rate was over 85% in both SGC-7901 and MGC-803 cells (Fig. 2D-E).

CLIC1 inhibits apoptosis of GC in vitro

Apoptosis was detected using FCM. The apoptosis rate of the knock down (KD) group was significantly higher than that of the CON group in SGC-7901 (25.52% vs 12.67%, $P < 0.05$) and

Fig. 1. Expression of the CLIC1 protein in GC and adjacent noncancerous tissue of GC patients. Positive staining for CLIC1 was found mainly in the GC-adjacent noncancerous tissue (200 $\times$) (A, B). Expression of CLIC1 staining between GC and adjacent noncancerous tissue (C).

MGC-803 cells (17.90% vs 6.09%, $P < 0.05$). There was no significant difference in the apoptosis rate between the CON and NC groups ($P > 0.05$) (Fig. 3A–D). This suggests that *CLIC1* silencing promotes the apoptosis of GC cells. We further detected the expression levels of apoptosis-related proteins (Bcl-2 and survivin), and the results showed that *CLIC1* silencing significantly suppressed Bcl-2 protein expression in both SGC-7901 and MGC-803 cells ($P < 0.05$), while the survivin protein expression was not significantly different between the KD and CON groups in both SGC-7901 and MGC-803 cells ($P > 0.05$) (Fig. 3E–F). Moreover, the expression levels of these proteins were similar between the CON and NC groups ($P > 0.05$) (Fig. 3E–F).

CLIC1 promotes GC cell migration and invasion in vitro

The migration assay revealed that the numbers of both SGC-7901 and MGC-803 cells crossing the membrane in the KD group were reduced significantly when compared with CON group cells (118.33 ± 8.51 vs 181.67 ± 11.72 , 108.67 ± 8.33 vs 185.00 ± 11.00 ; $P < 0.05$)

Table 1. Association of expression of CLIC1 with clinicopathological characteristics in gastric cancer [n (%)]. Note: #The 7thTNM Classification of Malignant Tumors proposed by the AJCC/UICC

Variable	Total patients(n=54)	Positive No.(%)	P
Sex			
Female	16	9(56.3)	0.762
Male	38	24(63.2)	
Age, years			
≤58 (median age)	27	15(55.6)	0.577
>58 (median age)	27	18(66.7)	
Size (maximal diameter)			
<5cm	29	17(58.6)	0.783
≥5cm	25	16(64.0)	
Lymph node metastasis			
Yes	39	25(64.1)	0.541
No	15	8(53.3)	
Distant metastasis			
Yes	17	12(70.6)	0.001
No	37	11(29.7)	
Pathological type			
low differentiated adenocarcinoma	34	20(58.8)	0.775
other	20	13(65.0)	
TNM stage #			
Stage I	15	8(53.3)	0.067
Stage II	8	2(25.0)	
Stage III	15	12(80.0)	
Stage IV	16	11(68.8)	
TNM stage #			
Stage I, II	23	10(43.4)	0.028
Stage III, IV	31	23(74.2)	

(Fig. 4A–B, 4E), and the migration inhibition rates were 34.87% and 41.26%, respectively. The invasion assay also displayed similar findings (86.00 ± 6.00 vs 156.67 ± 14.84 , 81.33 ± 5.03 vs 168.33 ± 17.39 ; $P < 0.05$) (Fig. 4C–D, 4F), and the invasion inhibition rate was 45.11% and 51.68% in SGC-7901 and MGC-803 cells, respectively. In conclusion, down-regulating CLIC1 expression inhibits the migration and invasion abilities of SGC-7901 and MGC-803 cells.

Protein mass spectrometry analysis of GC cells SGC-7901 after CLIC1 silencing

We set the protein content ratio of SGC-7901 and SGC-7901-KD more than 2.0 or less than 0.5 as differentially expressed proteins, and then we used SEQUEST Cluster software to search the Uniprot, IPI and NCBI data bases. The results revealed that there were 36 up-regulated and 18 down-regulated differentially expressed proteins from the SGC-7901 and SGC-7901-KD groups after CLIC1 silencing, including two ITGs, ITGα1 and ITGα3 (Tables 2

Fig. 2. The sequence diagram of the lentivector (segment) and transfection of GC cells. The yellow mark indicates the CLIC1-siRNA sequence (A). Transfection efficiency of GC cell lines SGC-7901 (100×) (B) and MGC-803 (100×) (C). CLIC1 mRNA expression levels were significantly suppressed in SGC-7901 cells (D) and MGC-803 cells (E) after lentivirus-mediated CLIC1-siRNA transfection (* $P < 0.05$ vs blank control and negative control groups).

Fig. 3. Apoptosis and apoptosis-related protein expression levels in GC cells after CLIC1 silencing. FCM showed that the apoptosis rate of SGC-7901 and MGC-803 cells increased significantly after CLIC1 silencing (* $P < 0.05$ vs blank control and negative control groups) (A-C). The CLIC1 and Bcl-2 expression levels were significantly suppressed (* $P < 0.05$ vs blank control and negative control groups), while the survivin expression level remained unchanged (* $P > 0.05$ vs blank control and negative control groups) in SGC-7901 and MGC-803 cells after CLIC1 silencing (D-E).

and 3). Then, we submitted integrin genes, CLIC1 and MAPK family genes into geneMANIA (<http://genemania.org/>) for signal pathway prediction to explore whether CLIC1 is associated with integrins and the MAPK signaling pathway. The results showed that CLIC1, ITG α v, ITG α 1, ITG α 3, ITG β 1, EKR, AKT1 and p38 are in the same signal network (Fig.5), which suggests there may be regulatory relationships among them. We performed protein mass spectrometry analysis in one of the GC cell lines, and the above hypothesis was confirmed in the following experiments in both of the SGC-7901 and MGC-803 cell lines.

CLIC1 silencing affected the expressions of ITGs

Our study showed that the *CLIC1* mRNA expression was significantly inhibited in both KD groups of SGC-7901 and MGC-803 cells ($P < 0.05$). *CLIC1* silencing significantly increased *ITG α 1* mRNA expression, but inhibited the expression of *ITG α 3*, *ITG α v* and *ITG β 1* mRNA ($P < 0.05$) (Fig. 6A–B). Western blot assays also displayed similar findings (Fig. 6C–D).

CLIC1 silencing inhibited the subcutaneous tumor growth of GC

The subcutaneous tumors of the original generation were successfully constructed in the SGC-7901, SGC-7901-NC, SGC-7901-KD, MGC-803 and MGC-803-NC groups in about 2–3 weeks in BALB/C nude mice, but failed in the MGC-803-KD group (Fig. 7A–B). The subcutaneous tumor growth of the original generation of the SGC-7901-KD group was significantly inhibited after *CLIC1* silencing ($P < 0.05$) (Fig. 7A). Finally, the *CLIC1* silencing group (MGC-803-chol) and its negative control group (MGC-803-NC1) were successfully established as subcutaneous tumors of the original generation after using siRNA by special chemical modification (chol-siRNA). We found that the tumor growth of the MGC-803-chol group was slower than the MGC-803-NC1 group ($P < 0.05$) (Fig. 7C). Compared with the corresponding control group, the subcutaneous tumor growth of second generation was significantly inhibited in the SGC-7901-KD and the MGC-803-chol groups ($P < 0.05$) (Fig. 7D–E). After HE staining, the subcutaneous tumor tissues that had cells with irregular arrangements, varied sizes, large nuclei that were deeply stained, an increased nucleoplasm ratio, obvious nuclear atypia and pathologic mitosis were verified as GC cells (Fig. 7F–G).

Table 2. The up-regulated expression proteins of SGC-7901 after CLIC1 interference in the Human database

Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	114/115	114/115 Count	# AAs	MW [kDa]	calc. pI
B4E0X7	Zinc finger protein 512	1.63	1.82	5	1	1	1	0.125	1	385	44.1	9.82
P48775	Tryptophan 2,3-dioxygenase	0.00	2.46	1	1	1	1	0.145	1	406	47.8	6.93
Q01970-2	Isoform 2 of 1-phosphatidylinositol 4,5-	1.68	0.69	2	1	1	1	0.198	1	1167	131.1	5.88
Q8WXE9	Stonin-2	1.90	0.99	2	1	1	1	0.204	1	905	101.1	5.39
H0YGE4	Protein LOC728763 (Fragment)	1.73	4.10	2	1	1	1	0.249	1	268	30.2	6.62
Q9Y277	Voltage-dependent anion-selective channel	4.62	6.36	3	1	2	3	0.282	1	283	30.6	8.66
P11274-2	Isoform 2 of Breakpoint cluster region	0.00	0.90	2	1	1	1	0.287	1	1227	137.6	7.21
J3QR09	Ribosomal protein L19	1.78	4.66	3	1	1	1	0.316	1	193	23.1	11.47
C9JNP9	Ubiquitin fusion degradation protein 1	1.85	5.03	6	1	1	1	0.342	1	159	17.5	4.75
P49207	60S ribosomal protein L34	0.00	7.69	1	1	1	1	0.351	1	117	13.3	11.47
F8W720	GMP synthase [glutamine-hydrolyzing]	7.82	8.92	2	4	4	5	0.362	4	594	65.9	7.47
J3QR71	Developmentally-regulated GTP-binding	0.00	12.66	5	1	1	1	0.364	1	158	16.9	9.06
A6NP16	Angiomotin	0.00	2.35	6	1	1	2	0.366	2	597	68.4	8.06
P18124	60S ribosomal protein L7	15.51	27.02	3	8	8	17	0.374	13	248	29.2	10.65
H0YHD8	Cysteine-rich protein 2 (Fragment)	0.00	18.27	4	1	1	1	0.375	1	104	11.2	8.38
P42765	3-ketoacyl-CoA thiolase, mitochondrial	1.95	2.77	1	1	1	1	0.386	1	397	41.9	8.09
Q14160	Protein scribble homolog	0.00	0.61	2	1	1	1	0.387	1	1630	174.8	5.07
E9PLH9	GDP-L-fucose synthase (Fragment)	2.06	9.24	4	1	1	1	0.389	1	119	12.9	8.03
F8VWC5	60S ribosomal protein L18	4.03	29.56	7	4	4	11	0.403	8	159	18.1	11.84
E7EPB3	60S ribosomal protein L14	1.69	9.68	2	1	1	2	0.404	2	124	14.5	10.21
P61254	60S ribosomal protein L26	0.00	5.52	1	1	1	2	0.404	2	145	17.2	10.55
P43490	Nicotinamide phosphoribosyltransferase	5.10	14.87	2	5	5	5	0.414	5	491	55.5	7.15
Q5T7N2	LINE-1 type transposase domain-	2.31	0.92	1	1	1	2	0.425	1	865	98.8	4.93
P50238	Cysteine-rich protein 1	0.00	20.78	3	2	2	4	0.432	4	77	8.5	8.75
P56199	Integrin alpha-1	1.77	0.76	1	1	1	1	0.446	1	1179	130.8	6.29
Q9Y3U8	60S ribosomal protein L36	1.78	10.48	1	1	1	1	0.451	1	105	12.2	11.59
P07741-2	Isoform 2 of Adenine	4.03	32.84	6	4	4	6	0.455	5	134	14.5	7.34
C9J8U2	Nicotinate phosphoribosyltransferase	11.19	14.69	6	6	6	10	0.459	8	490	52.1	5.33
P31942-6	Isoform 6 of Heterogeneous nuclear	2.47	7.19	7	1	1	1	0.464	1	139	15.4	6.40
P16152	Carbonyl reductase [NADPH] 1	5.65	40.43	5	8	8	12	0.470	10	277	30.4	8.32
P36578	60S ribosomal protein L4	4.07	10.30	4	4	4	12	0.475	12	427	47.7	11.06
P49411	Elongation factor Tu, mitochondrial	3.61	15.71	1	5	5	5	0.479	5	452	49.5	7.61
P09874	Poly (ADP-ribose) polymerase 1	0.00	0.69	1	1	1	1	0.489	1	1014	113.0	8.88
E9PN89	Heat shock cognate 71 kDa protein	148.16	57.69	1	1	18	128	0.489	1	312	34.8	7.53
P63173	60S ribosomal protein L38	0.00	14.29	1	1	1	1	0.495	1	70	8.2	10.10

Table 3. The down-regulated expression proteins of SGC-7901 after CLIC1 interference in the Human database

Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	114/115	114/115 Count	# AAs	MW [kDa]	calc. pI
A6NNI4	CD9 antigen	4.26	6.29	3	1	1	4	2.022	2	159	17.8	6.29
O75874	Isocitrate dehydrogenase [NADP] cytoplasmic	18.25	21.50	4	7	7	14	2.039	10	414	46.6	7.01
Q02539	Histone H1.1	52.51	27.91	2	2	6	39	2.049	3	215	21.8	10.99
Q9BT09	Protein canopy homolog 3	9.40	12.59	1	3	3	5	2.136	5	278	30.7	5.49
Q9UNZ2-4	Isoform 2 of NSFL1 cofactor p47	8.35	25.37	7	6	6	10	2.168	5	339	37.3	5.11
Q96P20-2	Isoform 1 of NACHT, LRR and PYD domains-containing protein 3	0.00	2.28	4	1	1	1	2.182	1	922	105.9	6.64
K7EMU3	Integrin alpha-3 (Fragment)	0.00	8.55	4	1	1	1	2.193	1	117	12.9	5.40
C9JYP1	Protein FAM107B (Fragment)	0.00	17.91	9	1	1	1	2.218	1	67	8.0	9.47
Q5RKY6	Exosome complex component MTR3	0.00	4.04	1	1	1	1	2.231	1	272	28.2	6.28
O94826	Mitochondrial import receptor subunit TOM70	2.04	2.47	1	2	2	2	2.252	2	608	67.4	7.12
B8ZZQ6	Thymosin alpha-1	98.37	14.02	6	4	4	61	2.281	57	107	11.8	3.81
Q9NNW7-4	Isoform 4 of Thioredoxin reductase 2, mitochondrial	2.26	3.97	9	1	1	1	2.525	1	277	30.3	6.28
P17096-2	Isoform HMG-Y of High mobility group protein HMG-1/HMG-Y	6.49	34.38	4	3	3	5	2.747	3	96	10.7	10.32
P12277	Creatine kinase B-type	31.70	30.18	6	9	9	21	2.842	18	381	42.6	5.59
P04264	Keratin, type II cytoskeletal 1	10.93	9.78	3	5	6	9	3.007	5	644	66.0	8.12
F8WBE5	Transferrin receptor protein 1, serum form	1.76	17.28	2	1	1	1	3.204	1	81	8.9	5.33
Q9H4G0-3	Isoform 3 of Band 4.1-like protein 1	0.00	3.50	4	2	2	2	3.717	1	772	86.5	5.81
Q9Y275-2	Isoform 2 of Tumor necrosis factor ligand superfamily member 13B	2.03	3.76	2	1	1	1	4.452	1	266	29.1	6.90

Orthotopically transplanted GC tumors were successfully established

The mice were sacrificed after feeding for several weeks. The orthotopically transplanted GC tumors were successfully constructed, and the tumor volume and mass of the SGC-7901-KD and MGC-803-chol groups were significantly lower than those of the corresponding

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control groups (Fig. 8A-F). The CLIC1 protein expression detected by WES technology was significantly reduced in the SGC-7901-KD and MGC-803-chol groups (Fig. 8G-I).

CLIC1 inhibited the apoptosis of orthotopically transplanted GC tumors

The apoptosis of orthotopically transplanted GC tumors was detected using TUNEL by DAB and fluorescence staining. The apoptosis rate after CLIC1 silencing was significantly higher than that of the CON group in SGC-7901 and MGC-803 cells (21.92% vs 2.12% and 26.50% vs 3.35%, respectively, $P<0.05$) (Fig. 9A). When fluorescence staining was used, the apoptosis rate was similar to that measured by DAB staining ($P<0.05$) (Fig. 9B). We further detected the

Fig. 5. The CLIC1 regulatory relationship with integrin genes and MAPK family genes by geneMANIA for signal pathway prediction.

Fig. 6. The mRNA and protein expression levels of integrins from SGC-7901 and MGC-803 cells in vitro after CLIC1 silencing. CLIC1 silencing significantly increased ITG α 1 mRNA expression, but inhibited the ITG α 3, ITG α v and ITG β 1 mRNA expression levels (* P <0.05 vs blank control and negative control groups) in SGC-7901 and MGC-803 cells (A-B). The protein expression levels were similar to the mRNA levels (D-E) (* P <0.05 vs blank control and negative control groups).

Fig. 7. The subcutaneous tumor growth of SGC-7901 and MGC-803 cells in vivo after CLIC1 silencing and histopathological results. The growth of primary subcutaneous tumors of the SGC-7901-KD group was significantly inhibited ($*P<0.05$, knock down group vs blank control and negative control groups) (A), but the primary subcutaneous tumors of MGC-803-CLIC1-KD failed to establish (B). The growth of primary subcutaneous tumors of the MGC-803-CLIC1-chol group were significantly inhibited ($*P<0.05$, knock down group vs corresponding negative control group) (C). The subcutaneous tumor growth of the second generation was also significantly inhibited in the SGC-7901-CLIC1-KD and MGC-803-CLIC1-chol groups ($*P<0.05$) (D-E). After HE staining, the subcutaneous tumor tissues of SGC-7901 and MGC-803 groups were verified for GC by HE staining (400 $\times$) (F-G).

expression levels of apoptosis-related proteins (Bcl-2 and Bax), and the results showed that CLIC1 silencing significantly suppressed Bcl-2 and increased Bax protein expression in both SGC-7901 and MGC-803 cells ($P<0.05$) (Fig. 9C-E).

Metabolic rate of the orthotopically transplanted tumors was reduced after CLIC1 silencing

Compared with the corresponding control group, PET-CT showed that the metabolic rate of the orthotopically transplanted tumors was significantly reduced in the SGC-7901-KD and MGC-803-chol groups ($P<0.05$), but distant metastasis was not observed in any of the groups (Fig. 10A-D).

CLIC1 silencing affected the relative protein expression levels of ITGs and signaling pathways

WES technology showed that the protein expression of ITG α 1 was increased, but ITG α 3, ITG α 5, ITG α v and ITG β 1 were down-regulated in orthotopically transplanted tumors of the SGC-7901-KD and MGC-803-chol groups after CLIC1 silencing ($P<0.05$) (Fig. 11A-B). Moreover, down-regulating CLIC1 also affected the expression levels of signaling pathway proteins. Our results showed that the protein expression levels of p-ERK and p-AKT in orthotopically transplanted tumors from the SGC-7901-KD and MGC-803-chol groups after CLIC1 silencing were significantly lower than those of the control groups ($P<0.05$), but the expression level of p-p38 was increased ($P<0.05$). The expression levels of ERK, AKT and p38 were similar to those of the control groups ($P>0.05$) (Fig. 11A&C).

Discussion

Our results show that *CLIC1* silencing promoted GC cell apoptosis and inhibited migration and invasion *in vitro*. Moreover, inhibiting *CLIC1* affected the expression of ITGs at both the mRNA and protein levels. Furthermore, we demonstrated that *in vivo* *CLIC1* has a similar function, as *CLIC1* knockdown suppressed orthotopic xenograft tumor volume, weight, metabolism and lymphatic infiltration capacity, and promoted apoptosis. Additionally, inhibiting *CLIC1* not only effectively affected the expression of protein of ITGs, but also influenced AKT and MAPK pathways in orthotopically xenografted tumors in nude mice models.

CLIC1 is an intracellular ion channel that is involved in many important physiological processes in the body [3]. At present, *CLIC1* has been found to be closely related to many kinds of tumors. Several studies have shown that *CLIC1* is expressed at a low level in normal gastric tissues and highly expressed in GC tissues [14-16]. It has also been found that the expression of *CLIC1* in GC is closely related to the invasion and metastasis of lymph nodes and the infiltration of peripheral nerves, suggesting that *CLIC1* is a potential tumor marker [7]. In this study, we successfully established a recombinant lentiviral expression vector targeting *CLIC1* using RNAi, and then we established stable GC cell lines with low *CLIC1* expression. The inhibition rate of *CLIC1* in the GC lines SGC-7901-KD and MGC-803-KD was 89.3% and 93.8%, respectively, which provides a stable vector for further study of the relationship between GC and *CLIC1*.

Studies have confirmed that *CLIC1* is involved in the apoptosis of tumor cells. Kang et al. [17] showed that inhibiting the expression of *CLIC1* in glioma stem cells promoted the apoptosis of tumor cells. Our results showed that the apoptosis rate of GC cells after knockdown of *CLIC1* significantly increased, suggesting that *CLIC1* silencing is able to promote the apoptosis of GC cells. In addition, we measured the expression of apoptosis-related proteins (Bcl-2 and survivin) to elucidate the anti-apoptotic mechanisms of *CLIC1* in each group. Notably, the expression levels of these two proteins were the same after *CLIC1* silencing. In the silenced groups, Bcl-2 expression decreased while survivin expression remained unchanged.

Migration and invasion are the basic processes of metastasis in malignant tumor cells. Reports have shown that *CLIC1* silencing significantly decreased the migration and invasion of

Fig. 8. The tumor growth and *CLIC1* protein expression of orthotopically transplanted tumors. The tumor volume and mass of orthotopically transplanted tumors in the SGC-7901-KD and MGC-803-chol groups were significantly lower than corresponding control groups (* $P < 0.05$) (A-F). With corresponding control groups, the *CLIC1* protein expression of orthotopically transplanted tumors was also significantly reduced in the SGC-7901-KD and MGC-803-*CLIC1*-chol groups (* $P < 0.05$) (G-I).

Fig. 9. The apoptosis of orthotopically transplanted tumors of GC and the expression levels of apoptosis proteins. The apoptosis of orthotopically transplanted GC tumors in both the SGC-7901-KD and MGC-803-chol groups were higher than those of the control group (* $P < 0.05$) (A–B). CLIC1 silencing significantly suppressed Bcl-2 and increased Bax protein expression levels in both SGC-7901 and MGC-803 cells (* $P < 0.05$) (C–E).

colon cancer and prostate cancer cells [5, 6], while *CLIC1* over-expression markedly increased the migration and invasion of hepatocellular carcinoma cells [18]. Our results show that the migration and invasion of SGC-7901KD and MGC-803KD cells significantly decreased after inhibition of *CLIC1* expression, which is consistent with the results of previous studies.

The mechanism of how *CLIC1* is involved in apoptosis, invasion and metastasis of GC cells remains unclear. ITGs play an important role in the apoptosis, invasion and metastasis of GC [19, 20]. It is possible that *CLIC1* interacts with ITGs to regulate the progression of GC. In order to verify this hypothesis, we used iTRAQ technology to analyze the differentially expressed proteins of SGC-7901 GC cells before and after inhibiting the expression of *CLIC1* [21]. We found that ITGs were differentially expressed, and verification experiments showed that

Fig. 10. The metabolic rate of primary orthotopically transplanted tumors by PET-CT. The metabolic rate of primary orthotopically transplanted tumors after *CLIC1* silencing was significantly reduced in both the SGC-7901 and MGC-803 groups (* $P < 0.05$) (A–D).

the expression of ITG α 3, ITG α v and ITG β 1 in the two GC cell lines were down-regulated at both the mRNA and protein level, but the expression of ITG α 1 was up-regulated. Other studies have investigated the level of ITGs in various tumors. Tanaka and Fukuzawa [22] found that the invasiveness of human neuroblastoma cells was inhibited by up-regulating ITG α 1. Additionally, Nakada et al. [23] reported that ITG α 3 is over-expressed in glioma stem-like cells and promotes invasion. Furthermore, evidence shows that over-expression of ITG α v is closely associated with the metastasis and progression of some cancers [24, 25].

Specifically, Yim et al. [26] showed that ITG α 1 was significantly associated with the occurrence of GC. In addition, Fukuda et al. [20] found that ITG α 1 was highly expressed in GC cells, and using a specific blocking agent, the adhesion of GC cells decreased significantly. In this study, we found that ITG α 1 was over-expressed, and migration and invasion was inhibited, results that are corroborated by the findings of Fukuda et al.

Studies have found that ITG α 3 is an important component of the receptors of the basement membrane, which is also involved in the reorganization of the cytoskeleton, and mediates the movement of GC cells to distant metastasis. Moreover, there is a positive correlation between ITG α 3 and tumor TNM stage and lymph node metastasis [27]. Combined with the down-regulation of ITG α 3 in our study, GC cells appear to infiltrate the basement membrane and degrade the extracellular matrix, leading to the inhibition of distant metastasis, which is consistent with our results. ITG α v is over-expressed in human GC tissue and is associated with the five year survival rate of GC patients [28]. Additionally, studies have indicated that ITG α v affects lymph node metastasis and the formation of MMP-9, which could directly provide the driving force for cell migration, especially the migration of cancer cells through endocytosis exocytosis cycle [29, 30]. Combined with the down-regulation of ITG α v in our study, we speculate that this could lead to the decrease in invasion and metastasis of GC cells. ITG β 1 may be an independent prognostic factor for GC patients. It promotes angiogenesis, lymph node metastasis and

Fig. 11. The relative protein expression levels of integrins and signaling pathways after CLIC1 silencing in vivo by WES technology. In the SGC-7901 and MGC-803 groups, the protein expression of ITG α 1 was increased, but the integrins ITG α 3, ITG α 5, ITG α v and ITG β 1 were down-regulated in orthotopically transplanted tumors after CLIC1 silencing (* $P < 0.05$) (A–B). In the SGC-7901 and MGC-803 groups, the protein expression levels of p-ERK and p-AKT in orthotopically transplanted tumors after CLIC1 silencing were significantly lower than those of the control groups, but p-p38 was increased (* $P < 0.05$) and the ERK, AKT and p38 levels were similar to those of the control groups ($P > 0.05$) (A and C).

vascular invasion, which often indicates poor prognosis [31]. Inhibition of the expression of ITG β 1 inhibited the peritoneal metastasis of GC cells [32]. In the present study, the down-regulation of ITG β 1 was accompanied with a decreased in the metastasis of GC cells, similar to the aforementioned findings. Thus, *CLIC1* gene silencing affects the progression of GC cells *in vitro* by regulating the expression of ITG α 1, ITG α 3, ITG α v and ITG β 1.

Our *in vitro* results showing the effect of CLIC1 in GC cell lines prompted us to create an *in vivo* model of subcutaneous GC growth in BALB/c mice. We found that *CLIC1* knockdown suppressed orthotopic xenograft tumor volume, weight, metabolism and lymphatic infiltration capacity, but promoted apoptosis. Given that GC metastasis is the leading cause of GC related death, our observations may lead to important clinical developments. The effectiveness of CLIC1 *in vitro* has been described predominantly in cell lines, and these results are supported by Setti et al. [6, 17, 33]. Our data demonstrates the effectiveness of CLIC1 in clinically relevant GC models. However, the mechanism of how CLIC1 affects apoptosis, metabolism and metastasis in GC is not clear.

Based on the observation that CLIC1 promoted the apoptosis of human GC cells accompanied by down-regulation of the apoptotic protein BCL-2, we detected the expression levels of Bcl-2 and Bax in orthotopic xenograft tumor tissue. Bax is a homologous protein of Bcl-2, which promotes apoptosis. Bax and Bcl-2 can form an apoptotic regulatory system [34]. The results showed that Bcl-2 is down-regulated and Bax is up-regulated. Additionally, the inhibitory effect of CLIC1 on ITGs has been described in GC cell lines *in vitro*, and our research shows that ITG α 3, ITG α v and ITG β 1 were down-regulated, but ITG α 1 was up-regulated, consistent with what we observed *in vitro*. Hence, CLIC1 acts as a regulator of ITGs. On the other hand, ITGs may influence MAPK and AKT signaling in tumors [35-37]. Thus, we measured the expression of PI3K/Akt, MAPK/ERK, MAPK/p38 and MAPK/JNK in orthotopically xenografted tumors. The results showed that p-AKT and p-ERK were down-regulated, p-p38 was up-regulated and p-JNK was not expressed. Therefore, we propose the following as the molecular mechanism of CLIC1 regulation. First, the formation of a Bax homodimer (Bax-Bax) induces cell apoptosis, and when Bcl-2 is highly expressed, the more stable heterodimer (Bcl-2-Bax) is formed, which suppresses apoptosis. According to our results, with the silencing of *CLIC1*, Bax was overexpressed, while Bcl-2 was down-regulated in orthotopically transplanted tumors. We observed that relatively more Bax-Bax homodimers induced apoptosis. The phosphorylation of ERK is crucial for the activation of the MAPK/ERK pathway, and it was associated with apoptosis, metastasis of tumors [38, 39]. On the other hand, the phosphorylation of AKT is also crucial for the activation of the PI3K/AKT pathway, and it was associated with apoptosis, angiogenesis of tumors [40, 41]. We observed that CLIC1 blocked ERK-phosphorylation and AKT-phosphorylation, and both had reduced levels in orthotopically xenografted tumors. Studies have shown that ITG α v may be involved in the regulation of the MAPK/ERK and PI3K/AKT pathways, affecting tumor apoptosis and metastasis [40, 42]. In our study, the expression of ITG α v was down-regulated, while at the same time, ERK-phosphorylation and AKT-phosphorylation were down-regulated after silencing *CLIC1*. This suggests that interference with the MAPK/ERK and PI3K/AKT pathways might be responsible for the pro-apoptotic and metastatic effects of CLIC1 induced by ITG α v in orthotopically xenografted GC tumors. In addition, ITG α 3 was over-expressed in glioma stem-like cells and promoted invasion via MAPK/ERK [23]. The MAPK/p38 pathway is mainly associated with inflammation, stress and apoptosis, which are involved in the occurrence and development of GC by p38-phosphorylation activation in gastric epithelial cells [43, 44]. When the level of p38 phosphorylation is increased, the apoptosis of GC cells increases [45]. In our study, p38-phosphorylation was increased, and the apoptosis of orthotopically xenografted tumors also increased.

There are some limitations to our study, which we plan to address in the future. First, we only used iTRAQ to screen the differential proteomics of the SGC-7901 GC cell line. Second, the mechanism of the interaction between CLIC1 and ITGs protein family is unclear.

Conclusion

Our finds showed that siRNA-mediated down-regulation of CLIC1 expression can effectively inhibit the migration and invasion of GC *in vitro* and *in vivo*, promote apoptosis and alter the expression ITG family proteins (including ITG α 1, ITG α 3, ITG α v and ITG β 1) to different extents. Additionally, it regulates signaling pathway cascades. Thus, CLIC1 may play an important role in the progression of GC, and its mechanism may affect the expression of ITG family proteins, which leads to the subsequent regulation of the PI3K/AKT, MAPK/ERK and MAPK/p38 pathways.

Abbreviations

CLIC1 (Chloride intracellular channel 1); GC (Gastric cancer); RNAi (RNA interference); iTRAQ (isobaric tag for relative and absolute quantitation); ERK (extracellular regulated MAP kinase); p-ERK (phosphorylated extracellular regulated MAP kinase); AKT (AKT serine/threonine kinase); p38 (p38 kinase); WES (Protein Simple Wes).

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This study was approved by the Ethical Review Committee of the First Affiliated Hospital of Guangxi Medical University. Written informed consent was obtained from all patients.

Disclosure Statement

No conflict of interests exists.

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