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Expression of tight junction transmembrane protein Claudin-1 in gastric carcinoma and effects on tumor cell proliferation, invasion and migration

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Abstract

***Background**: Claudin-1 is involved in various cancers, but its expression and role in gastric carcinoma remain unclear. **Materials and Methods**: Gastric carcinoma and adjacent normal tissues were harvested from 60 patients. Claudin-1 expression was detected by RT-qPCR. The expressions in human gastric carcinoma MKN45, SGC7901 and MKN28 cells and immortalized human gastric epithelium GES-1 cells were determined by RT-qPCR and Western blotting. Claudin-1 was overexpressed in SGC7901 cells by lentiviral transfection, and they were divided into Control (untransfected), normal control (NC) (transfected with lentiviral vector) and Claudin-1 (transfected with Claudin-1 overexpression lentivirus) groups. The proliferation, invasion and migration of gastric carcinoma cells were detected through cell counting kit-8, Transwell and wound healing assays, respectively. The effects of Claudin-1 on the expressions of epithelial-mesenchymal transition (EMT) marker proteins E-cadherin and N-cadherin were detected by Western blotting. Ten 4-week-old male BALB/c nude mice were subcutaneously injected with lentivirus-treated SGC7901 cells to establish the transplanted tumor model, and the effect of overexpression of Claudin-1 was explored. **Results**: The expression of Claudin-1 in gastric carcinoma tissues was significantly lower than that in adjacent tissues ($P<0.05$). Overexpression of Claudin-1 significantly inhibited the proliferation, invasion and migration of SGC7901 cells, increased the expression of E-cadherin, and decreased that of N-cadherin ($P<0.05$). Overexpression of Claudin-1 in the mouse model significantly inhibited the growth of subcutaneous transplanted tumors ($P<0.05$). **Conclusion**: Claudin-1 has low expression in gastric carcinoma tissues. Overexpression of Claudin-1 inhibits the proliferation, invasion, migration, and EMT of gastric carcinoma cells, and subcutaneous tumorigenesis in nude mice.*

***Keywords**: proliferation, gastric cancer, invasion, Claudin-1, epithelial-mesenchymal transition*

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Introduction

Gastric carcinoma is one of the five most common causes of cancer death, seriously threatening people's health. Besides, gastric carcinoma relapses after surgery in nearly half of patients, and the 5-year overall survival rate is lower than 30% (1). Therefore, it is of great significance to find new therapeutic targets for gastric carcinoma. Claudins are a class of tight junction proteins. The changes in the expression of Claudins can be found in a variety of epithelium-derived cancers, and some Claudins can serve as biomarkers for evaluating the patients' prognosis (2). In addition to tight junction, Claudin-1,

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the first protein isolated from the Claudin family, is also involved in regulating various cellular activities, such as proliferation, migration and epithelial-mesenchymal transition (EMT) (3). Studies have shown that the expression of Claudin-1 declines in non-small cell lung cancer (4), pancreatic cancer (5), breast cancer (6), and esophageal cancer (7), but it rises in liver cancer (8). However, its expression and role in gastric carcinoma remain unclear. In the present study, therefore, the effects of Claudin-1 on the biological behaviors of gastric carcinoma cells were comprehensively explored through *in vivo* and *in vitro* experiments, so as to provide new ideas for the diagnosis and treatment of gastric carcinoma.

Materials and Methods

Clinical specimens

This study has been approved by the ethics committee of our hospital, and written informed consents have been obtained from all patients. A total of 60 cases of specimens of gastric carcinoma tissues and adjacent normal tissues (≥5 cm away from carcinoma tissues) were harvested from patients undergoing surgery in our hospital from January 2019 to December 2020, including 45 males and 15 females aged 43-75 years, with an average of (61.7±5.8) years. The tissue fragments were preserved in liquid nitrogen. Inclusion criteria: 1) Patients diagnosed as gastric cancer by gastroscopy and histopathology; 2) without major diseases. 3) without receiving preoperative neoadjuvant radiotherapy or chemotherapy. Exclusion criteria: 1) Patients with obvious inflammatory cell infiltration in adjacent tissues to cancer; 2) with gastric ulcer or erosion; 3) with other malignant tumors.

Laboratory animals

A total of 10, 4-week-old SPF male BALB/c nude mice were purchased from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, and routinely fed in the laboratory animal center of our hospital under good ventilation, (25±10)% and 12/12 h light/dark cycle for 1 week to adapt to the environment. The experiment started on the 2nd week.

Cells and reagents

The following cells and reagents were used: human gastric carcinoma MKN45, SGC7901 and MKN28 cell lines and immortalized human gastric epithelium GES-1 cells (Shanghai Cell Bank, Chinese Academy of Sciences), Claudin-1 mimics lentivirus and control lentiviral vector (Shanghai GenePharma Co., Ltd.), Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) (Gibco, USA), reverse transcription (RT) kits (Sigma, USA), Lipofectamine 3000 transfection reagent (Vector, USA), TRIzol kits (TaKaRa), MTS cell proliferation assay kits, Transwell assay kits and Western blotting kits (BD, USA), and goat anti-rabbit immunoglobulin G (IgG) H&L (HRP) (ab6721), rabbit anti-N-cadherin polyclonal antibody (ab76057), anti-E-cadherin antibody [4A2] (ab231303), goat anti-rabbit IgG H&L (Alexa Fluor® 647) pre-adsorbed secondary antibody (ab150083) and goat anti-mouse IgG H&L (FITC) (ab6785) (Abcam).

Detection of Claudin-1 expression in gastric carcinoma tissues and cells by RT-qPCR

The total ribonucleic acid (RNA) was extracted from specimens by TRIzol in each group. The following primers were used: Claudin-1 (FP: 5'-GAGACTACCACTGTCCCC-3', RP: 5'-AAAGAATCCTCAAAACCA-3'), β-actin (FP: 5'-GCCCATCTATGAGGGTTACGC-3', RP: 5'-GCTTTAGCCACGCTCGGTC-3'). The reaction conditions were as follows: pre-denaturation at 75°C for 2 min, 40 cycles × (denaturation at 90°C for 5 min, annealing at 60°C for 60 s, and extension at 72°C for 30 s). The relative

expression of Claudin-1 was calculated by $2^{-\Delta\Delta Ct}$ method. The assay was repeated for 3 times independently for each specimen.

Cell culture and transfection

MKN45, SGC7901, MKN28 and GES-1 cells were cultured in DMEM containing 10% FBS, and the medium was replaced every 2 days, followed by passage upon reaching about 90% confluence. SGC7901 cells in logarithmic growth phase were transfected with lentivirus using Lipofectamine 3000 by liposome-mediated method according to the instructions. Then the cells were divided into Control group (untransfected), normal control (NC) group (transfected with lentiviral vector) and Claudin-1 group (transfected with Claudin-1 overexpression lentivirus).

Detection of cell proliferation by cell counting kit-8 (CCK-8) assay

At 12 h after routine transfection, the cell density was adjusted, and the SGC7901 cells were inoculated into a 96-well plate (5×10^3 cells/well) and detected at 24, 48, 72 and 96 h, respectively, with 3 replicates for each well. After the old medium was discarded, the cells were added with 100 μL of complete medium and 10 μL of CCK-8 reagent, and incubated at 37°C for 2 h. Finally, the optical density (OD) at 450 nm was measured using a microplate reader, with a blank control for each plate.

Detection of cell invasion by Transwell assay

Matrigel (40 μL) was paved in the Transwell chamber and allowed to stand at 37°C for 1 h until coagulation for later use. The old medium was discarded and replaced with serum-free medium at 12 h before the experiment. After digestion, the cells were washed with 1× phosphate-buffered saline (PBS), counted and resuspended (5×10^5 cells). Then 200-250 μL of cell suspension was placed into the lower chamber, and 500 μL of complete medium was also added into the lower chamber (making sure there were no air bubbles between the lower-layer complete medium and the Transwell chamber), followed by incubation in an incubator for 24 h. Later, the cells were fixed with 4% paraformaldehyde, washed with PBS, and stained with 0.1% crystal violet dye at room temperature for 15 min in the dark. After rinsing with PBS, the internal Transwell chamber was wiped with cotton swabs, air dried upside down, observed, photographed and counted under a fluorescence microscope.

Detection of cell migration by wound healing assay

At 12 h after transfection, the cell density was adjusted, and the cells were inoculated into a 6-well plate (5×10^5 cells/well). Upon reaching more than 90% confluence, the plate was scratched in the middle with a 200 μL pipette tip, washed with PBS for 3 times and cultured with serum-free medium. The cell migration status was observed and photographed at 0 and 24 h under a general microscope, the images were analyzed and the cell migration rate was calculated.

Detection of expressions of Claudin-1 and EMT-related proteins by Western blotting

The specimens were collected in each group, the total protein was extracted from tissues and cells using total protein extraction kits, and the protein content was determined using BCA protein quantification kits. The protein samples were subjected to SDS-PAGE, transferred onto a polyvinylidene fluoride (PVDF) membrane, sealed with blocking buffer containing 5% BSA at room temperature for 2 h, and incubated with primary antibodies at an appropriate concentration at 4°C overnight. After the PVDF membrane was washed with buffer for 3 times the next day, the samples were incubated again with secondary antibodies at room temperature for 1 h, and the developing solution was added for exposure and development.

Subcutaneous tumorigenesis test of nude mice

After transfection, SGC7901 cells in logarithmic growth phase were harvested in NC group and Claudin-1 group, digested with trypsin, resuspended with serum-free medium, prepared into single cell suspension (5×10^7/mL), and subcutaneously injected into the left and right armpits of nude mice (left in NC group and right in Claudin-1 group). Four weeks later, the mice were sacrificed, and the subcutaneous transplanted tumors were taken out, washed with normal saline and weighed.

Statistical analysis

SPSS 19.0 software was used for statistical analysis, and GraphPad Prism 5.01 software was used for plotting. The comparison was made by *t* test between two groups. P<0.05 was considered statistically significant.

Results

Expression of Claudin-1 in gastric carcinoma tissues

The messenger RNA (mRNA) expression of Claudin-1 was detected by RT-qPCR in gastric carcinoma tissues and adjacent normal tissues. The results showed that the mRNA expression level of Claudin-1 in gastric carcinoma tissues was significantly lower than that in adjacent normal tissues (P<0.05) (**Figure 1**).

Correlations of Claudin-1 expression in gastric carcinoma tissues with clinicopathological parameters

Claudin-1 expression in gastric carcinoma tissues was not significantly correlated with age, gender, smoking history, drinking history, tumor size or tumor location (P>0.05), but had significant correlations with TNM stage, lymph node metastasis, depth of invasion, and degree of differentiation (P<0.05) (**Table 1**).

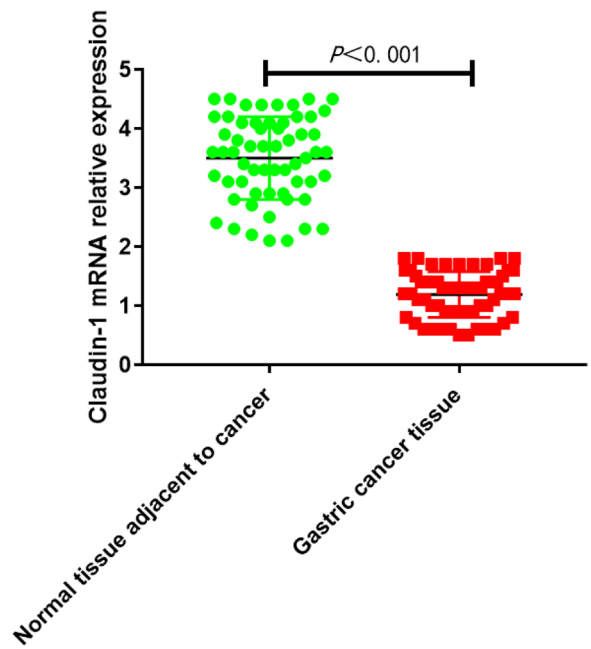

Fig. 1. MRNA expressions of Claudin-1 in gastric carcinoma and adjacent normal tissues detected by RT-qPCR.

Table 1. Correlations of Claudin-1 expression in gastric carcinoma tissues with clinicopathological parameters [n (%)]

Parameter	n	Claudin-1	P
Age (year)			>0.05
<65	32	1.11±0.25	
≥65	28	1.09±0.26	
Gender			>0.05
Male	45	1.12±0.28	
Female	15	1.08±0.25	
Smoking history			>0.05
Yes	26	1.11±0.22	
No	34	1.10±0.19	
Drinking history			>0.05
Yes	22	1.08±0.29	
No	38	1.13±0.31	
Tumor size (cm)			>0.05
≤5	28	1.08±0.23	
>5	32	1.15±0.11	
Tumor location			>0.05
Gastric antrum	33	1.08±0.13	
Stomach	11	1.12±0.24	
Gastric fundus, cardia	16	1.09±0.25	
TNM stage			<0.05
I~II	39	1.59±0.31	
III~IV	21	0.23±0.12	
Lymph node metastasis			<0.05
Yes	38	0.21±0.09	
No	22	1.82±0.31	
Invasion depth			<0.05
Mucosal layer	31	1.87±0.45	
Submucosa layer	29	0.19±0.07	
Differentiation degree			<0.05
Low	34	0.22±0.07	
Moderate/high	26	1.79±0.38	

Expression of Claudin-1 in gastric carcinoma cells

The expression of Claudin-1 was detected by RT-qPCR in human gastric carcinoma MKN45, SGC7901 and MKN28 cells and normal gastric epithelium GES-1 cells. It was found that the expression level of Claudin-1 in MKN45, SGC7901 and MKN28 cells declined compared with that in GES-1 cells, and it was the lowest in SGC7901 cells ($^{*}P<0.05$) (**Figure 2**). Therefore, SGC7901 cells were used for subsequent experiments.

Effect of Claudin-1 overexpression on proliferation of SGC7901 cells

After overexpression of Claudin-1 in SGC7901 cells, the transfection effect was detected using RT-qPCR. The expression of Claudin-1 in SGC7901 cells transfected with Claudin-1 overexpression lentivirus was about 4 times that in NC group, indicating that the lentivirus was successfully transfected into the SGC7901 cells. Then, the OD value at 450 nm in each group was measured using CCK-8 assay at 24, 48, 72 and 96 h. The results revealed that the cell proliferation ability significantly declined at 48, 72 and 96 h in Claudin-1 group compared with that in NC group, and the difference was statistically significant (P<0.05) (**Figure 3**). It can be seen that overexpression of Claudin-1 can greatly inhibit the proliferation ability of SGC7901 cells.

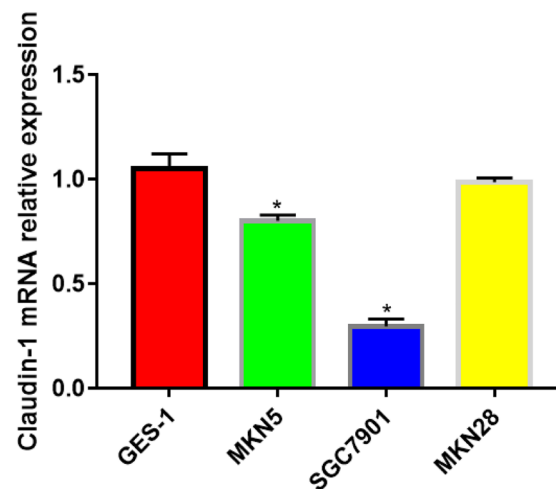

Fig. 2. MRNA expression of Claudin-1 in gastric carcinoma cells detected by RT-qPCR. $^{*}P<0.05$ *vs.* GES-1 cells.

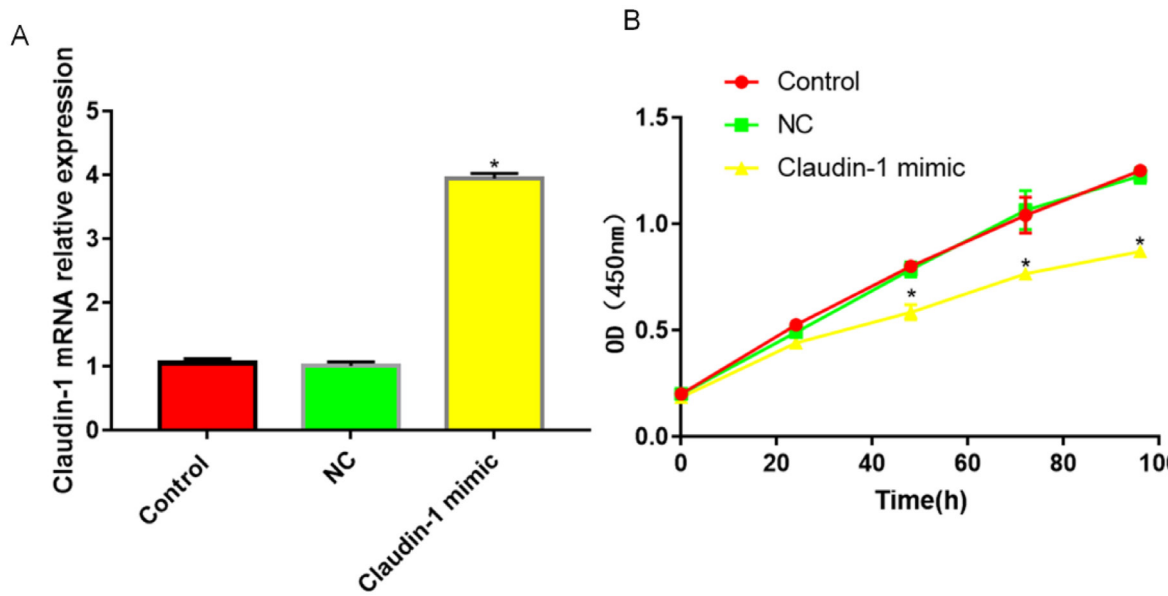

Fig. 3. Effect of Claudin-1 overexpression on proliferation of SGC7901 cells. A) Claudin-1 overexpression transfection effect detected by RT-qPCR, B) SGC7901 cell proliferation ability detected by CCK-8 assay. *P<0.05 *vs*. NC group.

Effect of Claudin-1 overexpression on invasion of SGC7901 cells

After overexpression of Claudin-1 in SGC7901 cells, the cell invasion ability in each group was detected by Transwell assay. The number of cells penetrating the Matrigel in Claudin-1 group was significantly smaller than that in the NC group, and the difference was statistically significant (P<0.05) (**Figure 4**). It can be inferred that the cell invasion ability becomes significantly weakened after overexpression of Claudin-1.

Effect of Claudin-1 overexpression on migration of SGC7901 cells

After overexpression of Claudin-1 in SGC7901 cells, the cell migration rate in each group was detected by wound healing assay. The results showed that both cell migration rate and wound healing rate significantly declined in Claudin-1 group compared with those in the NC group, showing statistically significant differences (P<0.05) (**Figure 5**). It can be inferred that the cell migration ability becomes significantly weakened after overexpression of Claudin-1.

Effects of Claudin-1 overexpression on EMT-related proteins E-cadherin and N-cadherin

It was found by Western blotting that Claudin-1 group had a higher expression of E-cadherin and a lower expression of N-cadherin than NC group (P<0.05) (**Figure 6**).

Effect of Claudin-1 overexpression on subcutaneous tumorigenesis in nude mice

SGC7901 cells transfected with lentivirus in NC group and Claudin-1 group were prepared into single cell suspension and subcutaneously injected into the left and right armpits of nude mice, and the effect of Claudin-1 overexpression on the subcutaneous tumorigenesis was observed. The volume of tumor formed in Claudin-1 group was significantly smaller than that in NC group. After the mice were sacrificed, the tumors on the

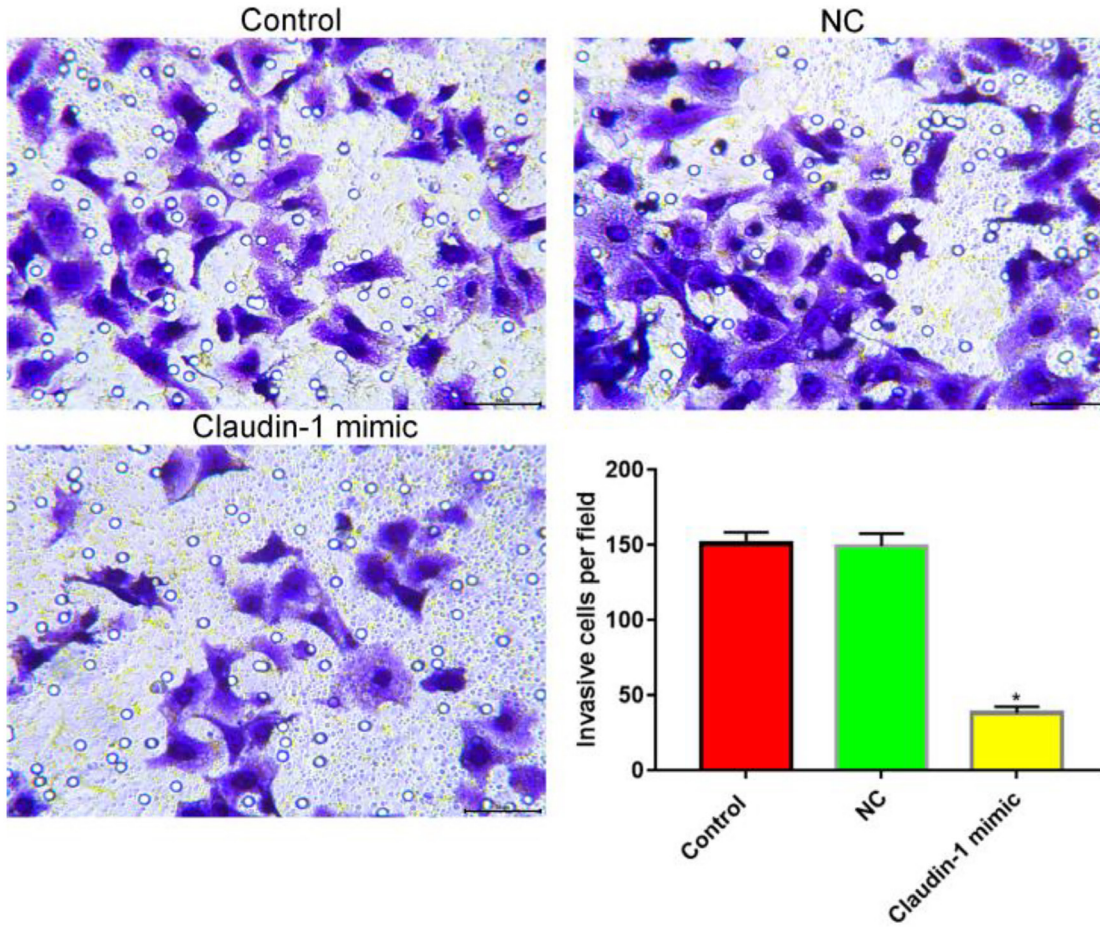

Fig. 4. Effect of Claudin-1 overexpression on invasion of SGC7901 cells. *P<0.05 *vs*. NC group.

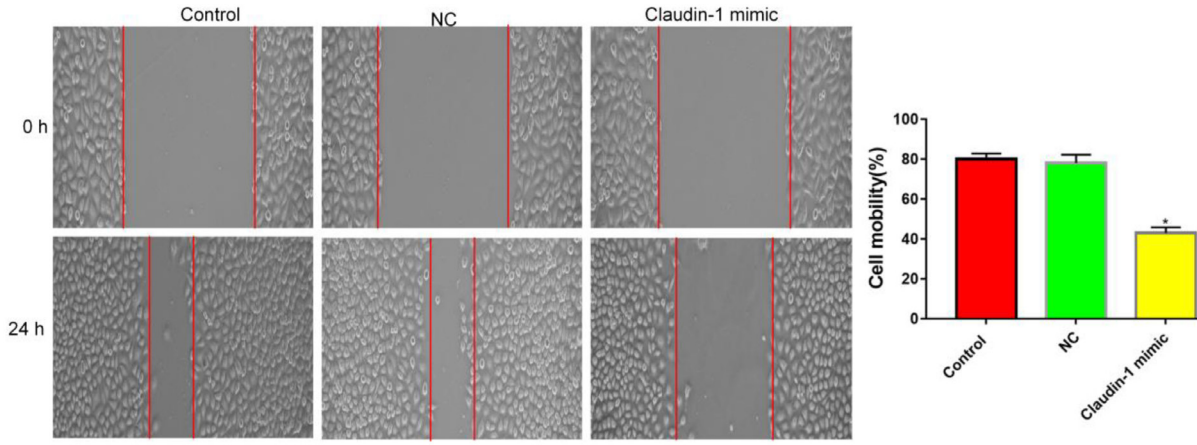

Fig. 5. Effect of Claudin-1 overexpression on migration of SGC7901 cells. *P<0.05 *vs*. NC group.

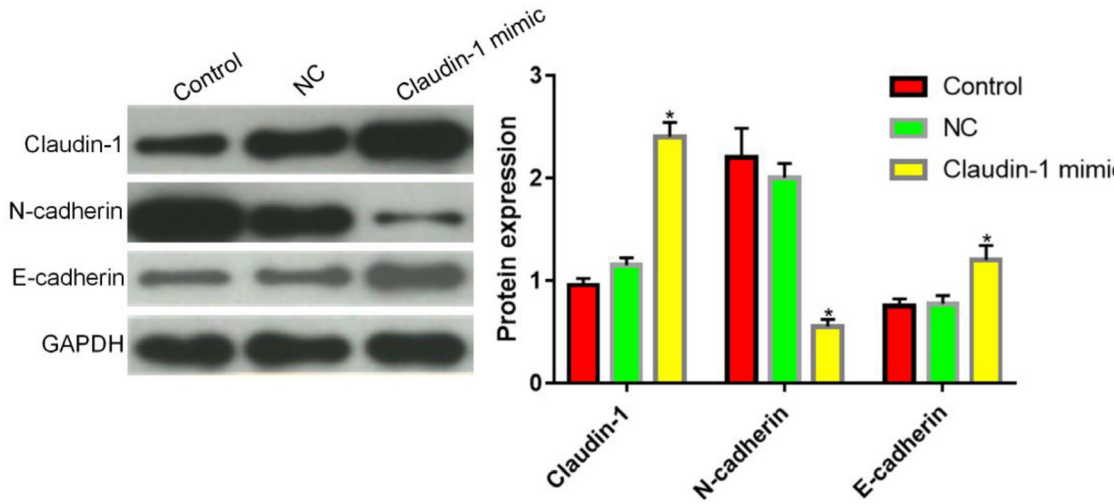

Fig. 6. Effects of Claudin-1 overexpression on EMT-related proteins E-cadherin and N-cadherin. *P<0.05 *vs*. NC group.

left and right sides were taken out and weighed. It was found that the tumor weight in Claudin-1 group was significantly smaller than that in NC group (*P<0.05) (**Figure 7**).

Discussion

Gastric carcinoma is one of the most common malignancies in the world, with a high mortality rate. The onset and progression of gastric carcinoma involve the regulation of multiple signals and genes, in which malignant epithelial transformation plays a key role (9). It has been found that the loss of intercellular junction is one of the important signs of epithelial malignant transformation (10). As a tight junction protein, Claudin-1 can regulate the transport of small

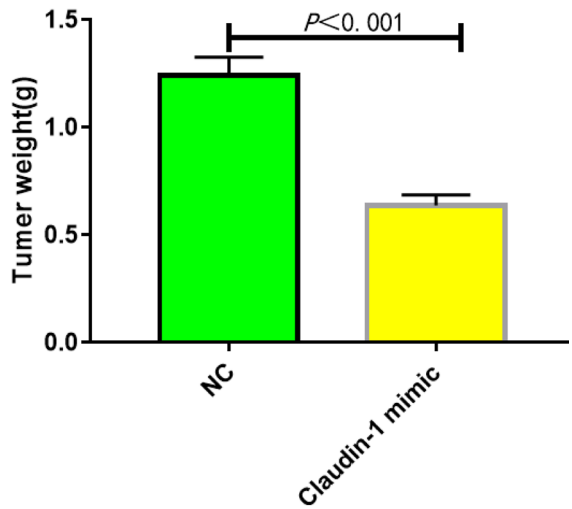

Fig. 7. Effect of Claudin-1 overexpression on subcutaneous tumorigenesis in nude mice. *P<0.05 *vs*. NC group.

ions between adjacent cells and maintain cell polarity, which is an important player in linking intercellular communication and keeping epithelial barrier function (11). According to several studies, the expression of Claudin-1 is absent in different tumors, and it regulates the proliferation, invasion and metastasis of tumor cells. For example, Ouban *et al* (12) found through analysis on the carcinoma and adjacent normal tissues of 50 patients with breast cancer that the expression of Claudin-1 significantly declined in carcinoma tissues, and it had significant negative correlations with tumor size, lymph node metastasis and tumor stage. Moldvay (4) and Nam *et al* (13) found that the high expression of Claudin-1 indicated a good prognosis of non-small cell lung cancer and colorectal cancer. Liu *et al* (5) confirmed that the expression of Claudin-1 was inhibited by ZIP4 in pancreatic cancer, thus facilitating invasion and metastasis of pancreatic cancer.

In the present study, gastric carcinoma and adjacent normal tissues were collected. The results of RT-qPCR showed that the expression of Claudin-1 in gastric carcinoma tissues was significantly lower than that in adjacent normal tissues, being consistent with the results of Wang *et al* (14) that Claudin-1 was weakly expressed in gastric carcinoma tissues. Herein, Claudin-1 expression in gastric carcinoma tissues was not significantly correlated with age, gender, smoking history, drinking history, tumor size or tumor location ($P>0.05$), but had significant correlations with TNM stage, lymph node metastasis, depth of invasion and degree of differentiation ($P<0.05$). The differential expression of Claudin-1 between gastric carcinoma tissues and adjacent normal tissues suggests the possible loss of Claudin-1 during malignant transformation of gastric carcinoma. Then the expression level of Claudin-1 was detected in human gastric carcinoma MKN45, SGC7901 and MKN28 cell lines and normal gastric epithelium GES-1 cells. It was found that the expression of Claudin-1 in gastric carcinoma cells significantly declined and it was the lowest in SGC7901 cells. Therefore, Claudin-1 was overexpressed in SGC7901 cells through gene transfection, and the effects on cell proliferation, invasion and migration were evaluated by CCK-8, Transwell and wound healing assays, respectively. The results showed that the proliferation, invasion and migration of SGC7901 cells were significantly suppressed after overexpression of Claudin-1.

Other studies have shown that Claudin-1 is also involved in the regulation on EMT (3, 15). EMT refers to the process in which epithelial cells gradually lose epithelial differentiation phenotype and acquire the mesenchymal phenotype, playing an important role in cancer invasion and metastasis (16). After polarization, the epithelial cells lose adhesion, the epithelial markers E-cadherin and ZO-1 are decreased, and the mesenchymal marker N-cadherin is increased. Deng *et al* confirmed that the loss of Claudin-1 could lead to EMT in gastric carcinoma, and EMT was the first step of gastric carcinoma metastasis (17). In the present study, the expressions of E-cadherin and N-cadherin were determined using Western blotting. The results revealed that compared with NC group, overexpressed Claudin-1 could inhibit the expression of N-cadherin and enhance the expression of E-cadherin, thereby reversing EMT. To sum up, Claudin-1 may suppress the invasion and metastasis of gastric carcinoma *via* reversing EMT.

In animal experiments, the cell lines stably overexpressing Claudin-1 were subcutaneously injected, and the subcutaneous tumorigenesis in nude mice was observed. The results revealed that after overexpression of Claudin-1, the tumor growth rate, volume and weight all declined. *In vivo* experiments confirmed that overexpression of Claudin-1 could inhibit the subcutaneous tumorigenesis in nude mice. Izraely *et al* also found that overexpression of Claudin-1 could not only

inhibit the invasion and migration of melanoma, but also reduce the tumorigenesis in nude mice (18).

In conclusion, it was proved through clinical specimens and *in vitro* cell experiments in this study that Claudin-1 was weakly expressed in gastric carcinoma, and overexpression of Claudin-1 could restrain the proliferation, invasion, migration, and EMT of gastric carcinoma cells. Further *in vivo* animal experiments showed that Claudin-1 inhibited the tumorigenesis in nude mice. These results strongly suggest that Claudin-1 plays a role in the occurrence and development of gastric carcinoma. Claudin-1 may serve as a new prognostic marker for gastric carcinoma, which inhibits malignant transformation by suppressing the proliferation, invasion and metastasis of gastric carcinoma cells. In the future, the molecular mechanism of Claudin-1 in the occurrence and development of gastric carcinoma will be further explored, so as to offer more sufficient evidence to clinical diagnosis and treatment.

Authors' contributions

Ang Cai designed this study and prepared this manuscript; Jun Lv and Zhuocui She collected and analyzed experimental data. All authors have approved the submission and publication of this manuscript.

Conflict of interest

There was no conflict in this work.

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