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CLDN18-ARHGAP26 function in gastric cancer and be a new therapeutic target by ABCG2 and ABCB1 pathway

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Abstract

Gastric cancer is one the most common human malignancies, with an increased incidence year by year. The underlying mechanisms of Claudin 18 (CLDN18) was involvement in patients with gastric cancer remain poorly understood. We therefore investigated the function of CLDN18 in patients with gastric cancer. Blood samples were collected from gastric cancer patients. CLDN18 and CLDN18 expression were measured using Microarray and qPCR. MTT assay, Transwell assay cells, LDH activity and Caspase-3/9 activity and Flow cytometry were used to measure the effects of ARHGAP26 and CLDN18 on cell growth in gastric cancer. We firstly found that CLDN18 expression were increased in patients with gastric cancer. Then, up-regulation of CLDN18 promoted cell growth in gastric cancer. Down-regulation of CLDN18 induced apoptosis in gastric cancer. Cancer-promoting genetic of CLDN18 is compromised by CLDN18-ARHGAP26 in gastric cancer cell. Down-regulation of ARHGAP26 rescues the effects of CLDN18-mediated tumor promotion effects on gastric cancer cell. CLDN18-ARHGAP26 mediated tumor suppressive effects on gastric cancer cells by ABCG2 and ABCB1 pathway. These results provide evidence that serum CLDN18-ARHGAP26 as a biomarker for tumor promoting genetic in gastric cancer via ABCG2 and ABCB1 pathway.

Keywords: CLDN18; gastric cancer; ARHGAP26; tumor suppressive; ABCG2; ABCB1.

Practical Application: The biomarker for tumor promoting genetic in gastric cancer.

1 Introduction

Gastric cancer is one the most common human malignancies, with an increased incidence year by year (Yu et al., 2019). According to the latest statistics, there are approximately 600,000 newly diagnosed gastric cancer patients globally each year (Yu et al., 2019). The dormant initial symptoms of gastric cancer and late diagnosis contribute to the high mortality of gastric cancer (Perez-Mendoza et al., 2018). Genetic and environmental variations have been confirmed to be the vital factors in the tumorigenesis and progression of gastric cancer (Choi et al., 2018).

Surgery is the most effective therapeutic approach for gastric cancer (Khan et al., 2017). However, the clinical symptoms of early stage gastric cancer is generally dormant, the majority of patients were initially diagnosed as intermediate and advanced gastric cancer (Jones, 1988). For patients with unresectable gastric cancer, the preferred treatment is transcatheter arterial chemoembolization (TACE) in clinical practice (Kang et al., 2018). Studies have shown that TACE can effectively improve the survival of patients with intermediate and advanced gastric cancer (Jones, 1988; Kang et al., 2018).

The Claudin 18.2 (CLDN18) family of transcription factors is one of the largest families of transcriptional regulators in cell, containing a total of more than 30 members (Wan et al., 2018). The majority of family members harbor various biological functions, including promoting cell proliferation and differentiation, inhibiting cell apoptosis and intercellular interactions, which exert regulatory effects on critical biological and pathological process (Hashimoto et al., 2019; Ismail et al., 2020; Li et al., 2020; Negrão et al., 2021). The migration, proliferation and differentiation of lumen morphology would lead to the angiogenesis of tumor (Balthazar et al., 2021; Hu et al., 2021; Khan et al., 2021). Recent studies have demonstrated that CLDN18, a member of CLDN family, is involved in regulation of angiogenesis of tumor, playing a vital role in the tumorigenesis and metastasis (Coati et al., 2019). Therefore, studies on the biological functions of CLDN18 family can provide theoretical guidance for clinical therapy of tumor to certain degree.

The association between ARHGAP26 attributed to various factors, demonstrates that in different stages of normal cell, progression and survival of tumors, many different genes and different variation formations may be involved, due to regulation and influence of different genes (Sahin et al., 2008). Activation or inactivation of ARHGAP26 signaling pathway regulates the effect on cell of its downstream target genes (Bartels et al., 2018; Chen et al., 2019). We therefore investigated the function of CLDN18 in patients with gastric cancer.

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2 Materials and methods

2.1 Patient specimens

Blood samples were collected from gastric cancer patients. Ethical approval was obtained from the Hospital Research Ethics Committee of the First Affiliated Hospital of Bengbu Medical College and written informed consent was obtained from all participants. Blood samples of 24 Healthy volunteers were collected, all blood samples were centrifuged at 1000 g for 10 min at 4 °C, and serum was collected and saved at -80 °C.

2.2 Microarray

Total RNA was extracted and purified using the mirVana™ miRNA Isolation Kit. Total RNA was amplified and labeled using the Low Input Quick Amp WT Labeling Kit and Labeled cRNA was purified using the RNeasy mini kit. Labeled cRNA was hybridized using the Gene Expression Hybridization Kit. The slides were washed in staining dishes with the Gene Expression Wash Buffer Kit. Shanghai Biotechnology Cooperation (Shanghai, P.R. China) was analyzed using Quantile algorithm in the limma package.

2.3 Reverse Transcription Polymerase Chain Reaction (RT-PCR) and real-time quantitative RT-PCR (qPCR)

qPCR for miRNA was performed to detect expression in serum using a QIAamp RNA Blood kit (Qiagen, Hilden, Germany). cDNA were synthesized from total RNA using QuantiFast SYBR Green PCR kit (Qiagen, Germany). Then, real-time quantitative RT-PCR analysis was detected by an Eppendorf Realplex2 Mastercycler (Eppendorf, Hamburg, Germany) using the Fast Start Universal SYBR Green Master (Applied Biosystems, Foster City, CA, USA).

2.4 Cell lines and transfection

Gastric cancer cell line GCIY cell was cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Gibco BRL, Grand Island, NY, USA) and 100 U/ml of penicillinestreptomycin in a humidified atmosphere of 5% CO2 at 37 °C. ARHGAP26plasmid, si- ARHGAP26 mimics, CLDN18 plasmid, si-CLDN18 mimics and negative mimics were purchased from GenePharma Company (Nanjing, People's Republic of China). GCIY cell were transfected with ARHGAP26 plasmid, si- ARHGAP26 mimics, CLDN18 plasmid, si-CLDN18 mimics and negative mimics using Lipofectamine 2000 Transfection Reagent (Invitrogen, CA, USA) according to the manufacturer's protocol. After transfection for 4 h, all cells were added with new DMEM for MTT assay at 24, 48 and 72 h; for Transwell assay cells at 24 h; for Flow cytometry, Western blotting, Immunohistochemistry and so on at 24 h.

2.5 MTT assay and Transwell assay cells

After 24, 48 and 72 h of transfection, cell was added with 20 μL of MTT for 4 h at 37 °C. DMSO was added into cell and cultured for 20 min at 37 °C after removing old DMEM. Transfection cell was assayed by measuring absorbance at 490 nm (OD values) using a Multiskan FC enzyme immunoassay analyzer (Thermo Fisher Scientific, Inc.).

1 × 105 cells/mL was seeded in the upper Transwell chamber (Corning Costar Corp, Corning, NY, USA) and the chambers were seeded into 24-well plates. 500 μL of DMEM with 10% FBS was added to the lower chambers and cultured for 48 h at 37 °C. The membranes were fixed in 4% paraformaldehyde and stained with 0.1% crystal violet. The number of cells penetrated across the membranes under inverted Olympus BX50 microscope (BX50, Olympus, Tokyo, Japan).

2.6 LDH activity and Caspase-3/9 activity

Total protein was extracted using RIPA lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) and protein concentration was measured using a bicinchoninic acid assay (BCA, Beyotime Institute of Biotechnology, Haimen, China). Caspase-3/9 activity was measured using a microplate reader by Caspase-3/9 activity kits at 405 nM.

2.7 Flow cytometry

Cell was washed with PBS and fixed with 4% paraformaldehyde for 15 min. Subsequently, the cells were stained with 5 μL Annexin V and 5 μL PI in 100 μL 1X binding buffer (BD Biosciences, Franklin Lakes, NJ, USA) for 15 min at room temperature in the dark. The cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and Image-ProPlus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

2.8 Western blotting

Cells were lysed with RIPA lysis buffer and protein concentrations assessed using a BCA protein assay kit. Equal amounts (50 μg) of protein were placed on 10% SDS-PAGE gels and blotted onto PVDF membranes. Membranes were blocked with 5% nonfat milk for 1 h at room temperature, and probed with CLDN18, ABCG2 and ABCB1, ARHGAP26 and GAPDH (Santa Cruz, Santa Cruz, CA, USA) at 4 °C overnight. The blots were washed with TBST for 1 h and then incubated with HRP-conjugated secondary antibody for 1 h at 37 °C. ECL substrates were used to visualize protein blank and Image Lab 3.0 (Bio-Rad Laboratories, Inc.) used to analzye protein blank.

2.9 Animal work

Animal experiments were approved by the Institutional Animal Care and Use Committee of Harrison International Peace Hospital. Nude mice (4-6 weeks) was injected with (1 × 106 cells per animal) of GCIY cell. The tumor volume was calculated using the following formula: volume (mm2) = length × width.

2.10 Statistical analysis

The data are expressed as the mean ± SD of three independent experiments. Statistical differences were compared using Student's t-test or one-way analysis of variance (ANOVA) and Tukey's post test. A p-value of < 0.05 was considered statistically significant.

3 Results

3.1 CLDN18-ARHGAP26

CLDN18 regulated cell proliferation and cell apoptosis in gastric cancer cells

The explored the effects of CLDN18 expression in patients with gastric cancer. As showed in Figure 1A-B, CLDN18 protein expression was up-regulated *in vitro* model of gastric cancer by CLDN18 plasmid, compared with negative group. Over-expression of CLDN18 promoted cell proliferation, cell migration and transferrer rate, and decreased apoptosis rate, LDH activity and caspase-3/9 activity levels *in vitro* model of gastric cancer, compared with negative group (Figure 1C-L). Next, si-CLDN18 mimics suppressed CLDN18 protein expression *in vitro* model of gastric cancer, compared with negative group (Figure 2A-B). Down-regulation of CLDN18 promoted cell proliferation, cell migration and transferrer rate, and reduced apoptosis rate, LDH activity and caspase-3/9 activity levels *in vitro* model of gastric cancer, compared with negative group (Figure 2C-L). Next, si--ARHGAP26 reduced ARHGAP26 protein expression, and increased tumor volume and weight, and reduced caspase-3/9 activity levels in vivo model, compared with negative group (Figure 3).

CLDN18 controls ARHGAP26 expression in gastric cancer cells

We determined the mechanism of CLDN18 on gastric cancer cell growth in vivo model. Q-PCR showed that CLDN18 expression was reduced in patient of gastric cancer, compared with normal group (para-carcinoma tissue, Figure 4A). The survival rate of CLDN18 high expression was higher than those of CLDN18 low expression (Figure 3B). Gene chip showed that CLDN18 up-regulate 31 genes and down-regulated 23 genes *in vitro* model of gastric

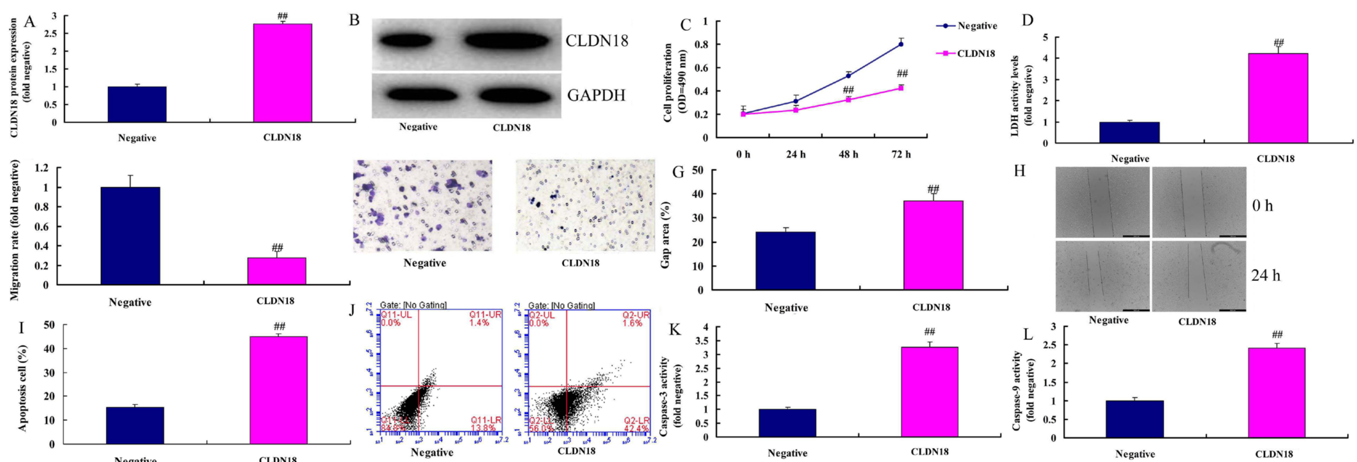

Figure 1. CLDN18 overexpression inhibits cell proliferation and induces cell apoptosis in gastric cancer cells. CLDN18 protein expression (A, B), cell proliferation (C), LDH activity (D), cell migration (E, F) and transferrer rate (G, H), cell apoptosis (I, J), caspase-3/9 activity (K, L). Negative, negative group; CLDN18, over-expression of CLDN18 group. ##P < 0.05 vs negative group.

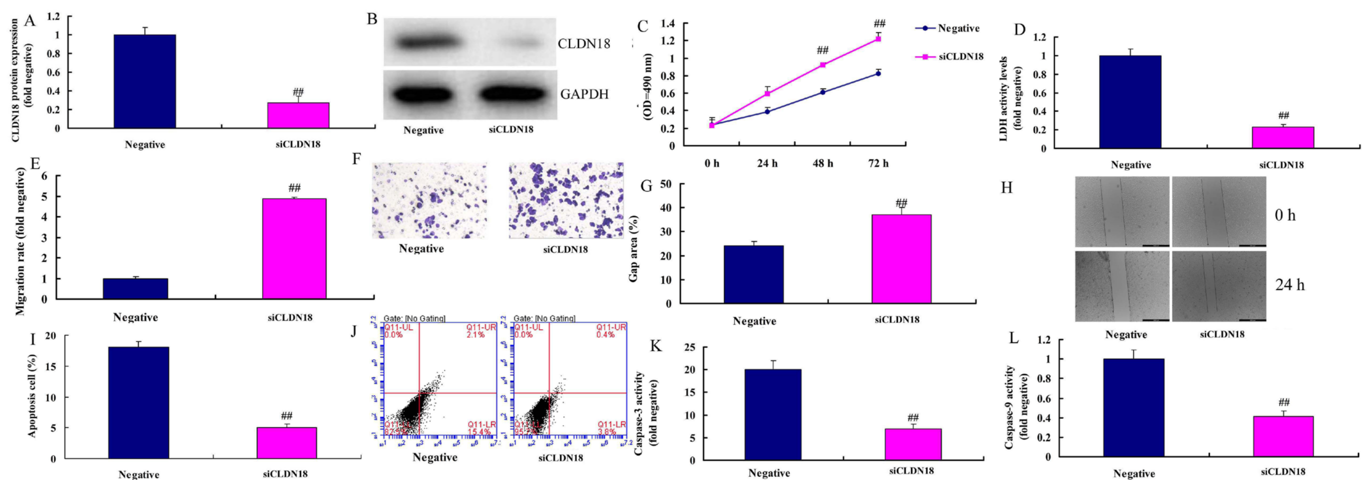

Figure 2. CLDN18 downregulation enhances cell proliferation and reduced cell apoptosis in gastric cancer cells. CLDN18 protein expression (A, B), cell proliferation (C), LDH activity (D), cell migration (E, F) and transferrer rate (G, H), cell apoptosis (I, J), caspase-3/9 activity (K, L). Negative, negative group; siCLDN18, down-regulation of CLDN18 group. ##P < 0.05 vs negative group.

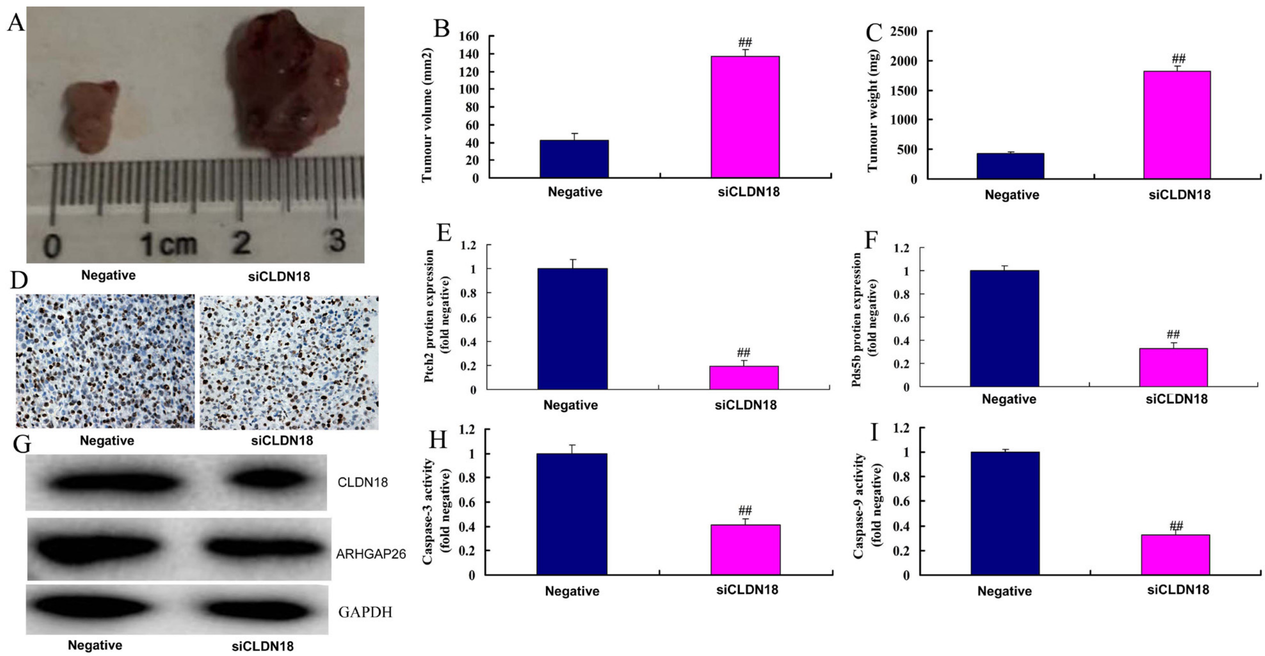

Figure 3. CLDN18 in gastric cancer patients and vivo model. Tumor volume and weight (A, B, C), Immunohistochemical for CLDN18 (D) in vivo model of gastric cancer, CLDN18 and CLDN18 protein expression (E, F, G), caspase-3/9 activity levels (H, I). Negative, negative group; siCLDN18, down-regulation of CLDN18 group. ##P < 0.05 vs negative group.

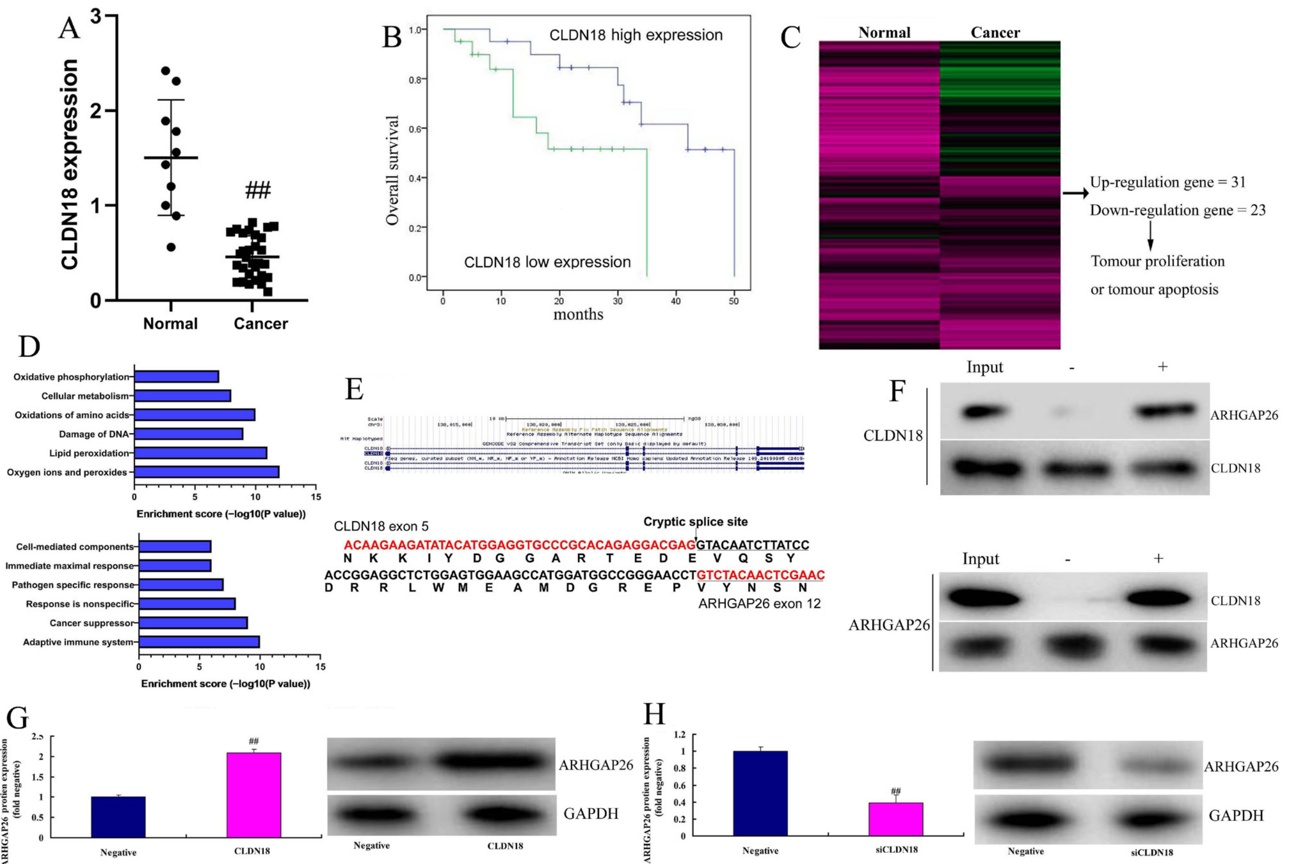

Figure 4. CLDN18 controls CLDN18 expression in gastric cancer cells. CLDN18 expression in patients with gastric cancer (A), overall survival (B), gene chip (C), network analysis graphics (D), IP for CLDN18 controls CLDN18 expression (E), CLDN18 protein expression (F, G) by over-expression of CLDN18; CLDN18 protein expression (H, I) by down-regulation of CLDN18. Negative, negative group; CLDN18, over-expression of CLDN18 group; siCLDN18, down-regulation of CLDN18 group. ##P < 0.05 vs negative group.

cancer (Figure 4C-D). These possible genes were used to analyze relevance of cancer treatment and we found that CLDN18 may control CLDN18 expression in gastric cancer cells and which was important target in cancer treatment of gastric cancer. In gastric cancer with CLDN18-ARHGAP26 fusions, the transcripts were joined by a cryptic splice site exon 5 of CLDN18 and the regular splice site of 12 of ARHGAP26 (Figure 4E). IP showed that CLDN18 combined with CLDN18 protein in gastric cancer cells (Figure 4F). Over-expression of CLDN18 induced CLDN18 protein expression *in vitro* model of gastric cancer, compared with negative group (Figure 4G). Down-regulation of CLDN18 suppressed CLDN18 protein expression *in vitro* model of gastric cancer, compared with negative group (Figure 4H).

CLDN18 regulates cell proliferation and induces cell apoptosis in gastric cancer cells

We determined the effects of CLDN18 on gastric cancer cell growth *in vitro* model. As showed in Figure 5A-B, CLDN18 protein expression was up-regulated *in vitro* model of gastric cancer by CLDN18 plasmid, compared with negative group. Over-expression of CLDN18 reduced cell proliferation, cell migration and transferrer rate, and increased apoptosis rate, LDH activity and caspase-3/9 activity levels *in vitro* model of gastric cancer, compared with negative group (Figure 5C-L). Next, si-CLDN18 mimics suppressed CLDN18 protein expression *in vitro* model of gastric cancer, compared with negative group (Figure 6A-B). Down-regulation of CLDN18 promoted cell proliferation, cell migration and transferrer rate, and reduced apoptosis rate, LDH activity and caspase-3/9 activity levels *in vitro* model of gastric cancer, compared with negative group (Figure 6C-L).

Downregulation of CLDN18 rescues the effects of ARHGAP26-mediated tumor suppressive effects on gastric cancer cells

The study determined the role of CLDN18 in the effects of CLDN18-mediated tumor suppressive effects on gastric cancer cells. Si-ARHGAP26 suppressed ARHGAP26 protein expression

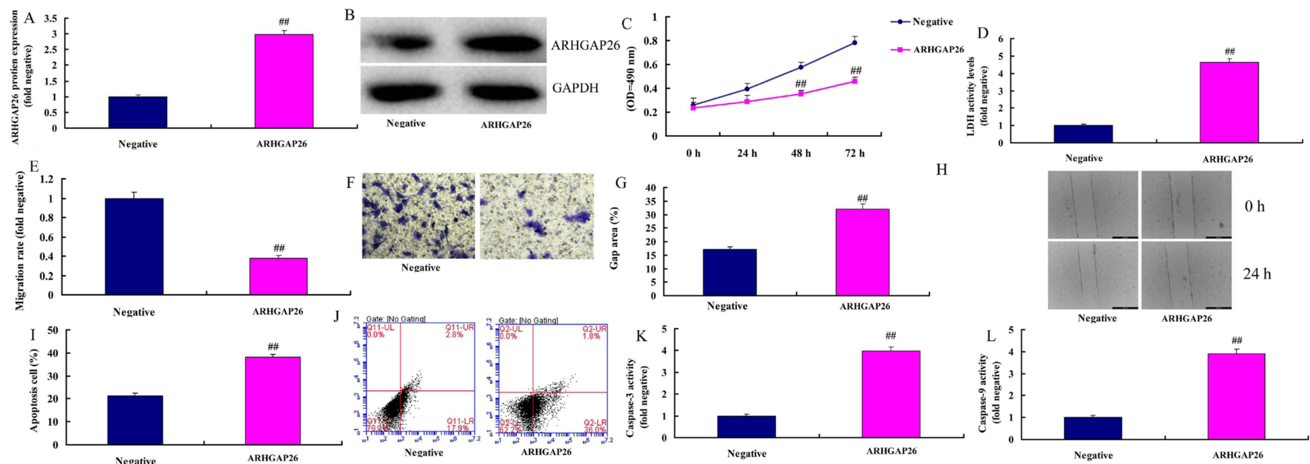

Figure 5. ARHGAP26 overexpression inhibits cell proliferation and induces cell apoptosis in gastric cancer cells. CLDN18 protein expression (A, B), cell proliferation (C), LDH activity (D), cell migration (E, F) and transferrer rate (G, H), cell apoptosis (I, J), caspase-3/9 activity (K, L). Negative, negative group; ARHGAP26, over-expression of ARHGAP26 group. ##P < 0.05 vs negative group.

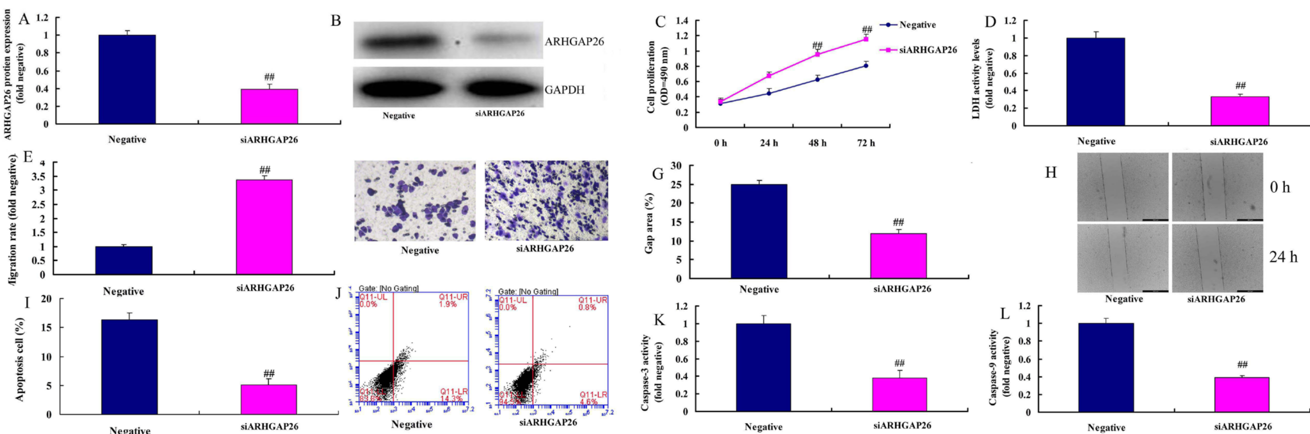

Figure 6. ARHGAP26 downregulation enhances cell growth and reduced cell apoptosis of gastric cancer cells. CLDN18 protein expression (A, B), cell proliferation (C), LDH activity (D), cell migration (E, F) and transferrer rate (G, H), cell apoptosis (I, J), caspase-3/9 activity (K, L). Negative, negative group; siARHGAP26, down-regulation of ARHGAP26 group. ##P < 0.05 vs negative group.

in vitro model of gastric cancer by over-expression of CLDN18, compared with over-expression of CLDN18 group (Figure 7A-B). The inhibition of ARHGAP26 promoted cell proliferation, cell migration and transferrer rate, and reduced apoptosis rate, LDH activity and caspase-3/9 activity levels *in vitro* model of gastric cancer by over-expression of CLDN18, compared with over-expression of CLDN18 group (Figure 7C-L).

The function of ARHGAP26 in gastric cancer patients and vivo model

The study explored the mechanism of ARHGAP26 on cell growth of gastric cancer. Q-PCR showed that ARHGAP26 expression was reduced in patient of gastric cancer, compared with normal group (para-carcinoma tissue, Figure 8A). The survival rate of ARHGAP26 high expression was higher than those of ARHGAP26 low expression (Figure 7B). Meanwhile, si-ARHGAP26 reduced ARHGAP26 protein expression, and increased tumor volume and weight, and reduced caspase-3/9 activity levels *in vivo* model, compared with negative group (Figure 8C-J).

ARHGAP26 reduced the effects of CLDN18 downregulation in vivo model

In vivo model, we found that over-expression of ARHGAP26 reduced the effects of CLDN18 downregulation on the promotion of tumor volume and weight *in vivo* model, compared with CLDN18 downregulation group (Figure 9A-C). ARHGAP26 plasmid induced CLDN18 protein expression *in vivo* model by CLDN18 downregulation, compared with CLDN18 downregulation group (Figure 9D-G). Next, over-expression of ARHGAP26 reduced the effects of CLDN18 downregulation on inhibition of caspase-3/9 activity levels *in vivo* model, compared with CLDN18 downregulation group (Figure 9H-I).

CLDN18-ARHGAP26 mediated tumor suppressive effects on gastric cancer cells by ABCG2 and ABCB1 pathway

The study explored the mechanism of CLDN18-ARHGAP26 on cell growth of gastric cancer. Gene chip showed that CLDN18 up-regulate 28 genes and down-regulated 35 genes *in vitro* model of gastric cancer (Figure 10A-B). Over-expression of ARHGAP26 induced ABCG2 and ABCB1 protein expressions and down-regulation of ARHGAP26 suppressed ABCG2 and ABCB1 protein expressions *in vitro* mode (Figure 10C-H). Meanwhile, over-expression of CLDN18 induced ABCG2 and ABCB1 protein expressions and down-regulation of CLDN18 suppressed ABCG2 and ABCB1 protein expressions *in vitro* mode (Figure 10I-N).

4 Discussion

With advance in recent years, the tumorigenesis of gastric cancer has been considered to be closely correlated with unbalanced control of cell cycle (Ihle, 2018). CLDN18 pathway is a classical

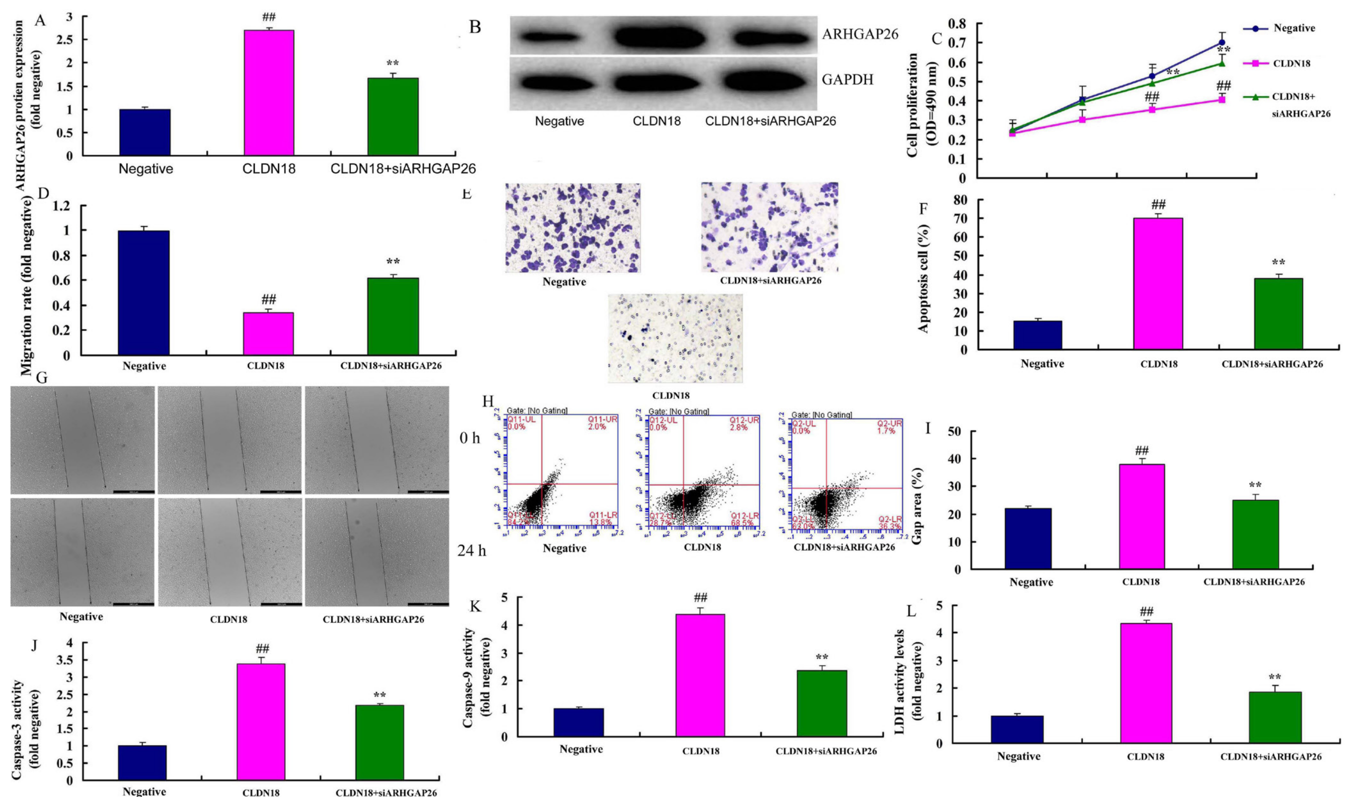

Figure 7. Downregulation of ARHGAP26 rescues the effects of CLDN18-mediated tumor suppressive effects on gastric cancer cells. CLDN18 protein expression (A, B), cell proliferation (C), LDH activity (D), cell migration (E, F) and transferrer rate (G, H), cell apoptosis (I, J), caspase-3/9 activity (K, L). Negative, negative group; CLDN18, over-expression of CLDN18 group; CLDN18+siARHGAP26, over-expression of CLDN18 and down-regulation of ARHGAP26 group. ##P < 0.05 vs negative group; **P < 0.05 vs over-expression of CLDN18 group

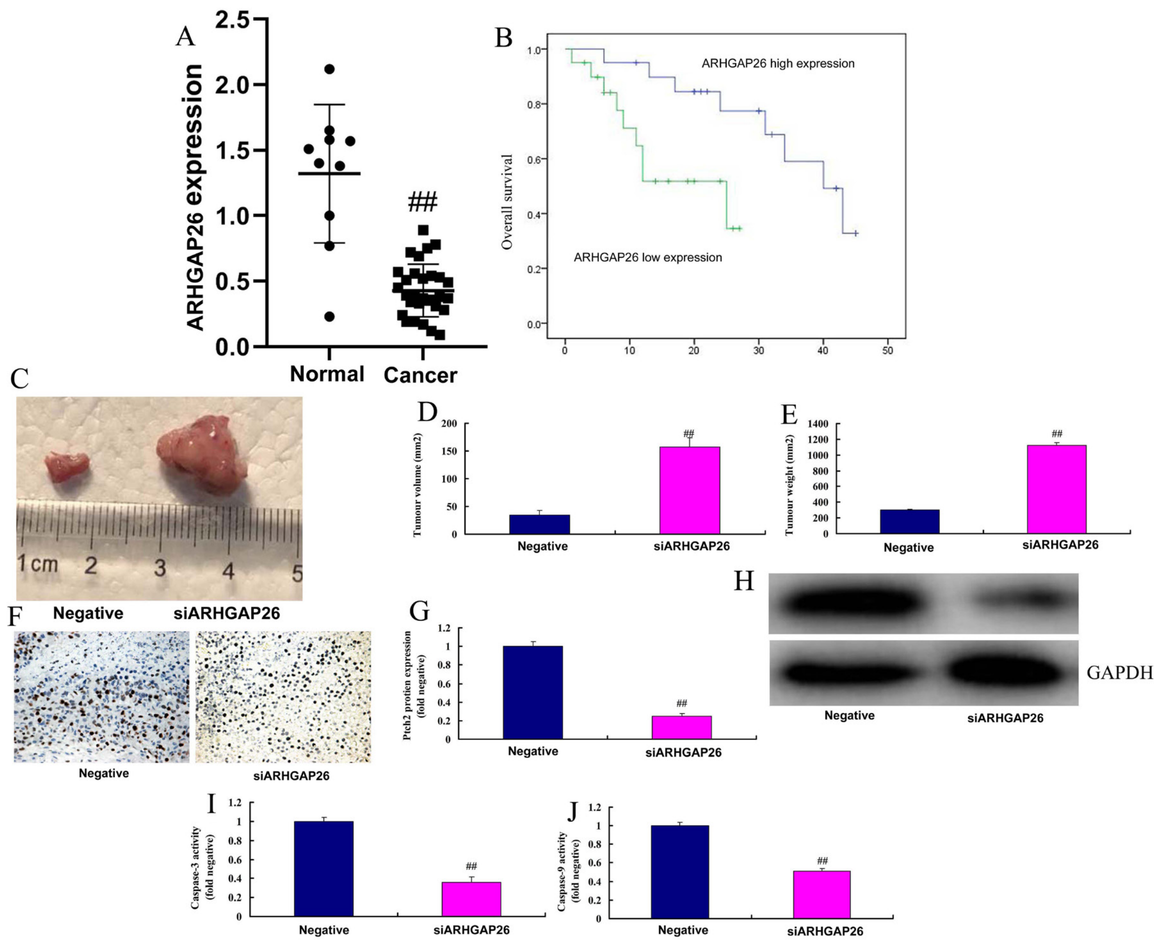

Figure 8. ARHGAP26 in gastric cancer patients and vivo model. ARHGAP26 expression in patients with gastric cancer (A), overall survival (B), tumor volume and weight (C, D, E), Immunohistochemical for ARHGAP26 (F) in vivo model of gastric cancer, ARHGAP26 protein expression (G, H), caspase-3/9 activity levels (I, J). Negative, negative group; siARHGAP26, down-regulation of ARHGAP26 group. ##P < 0.05 vs negative group.

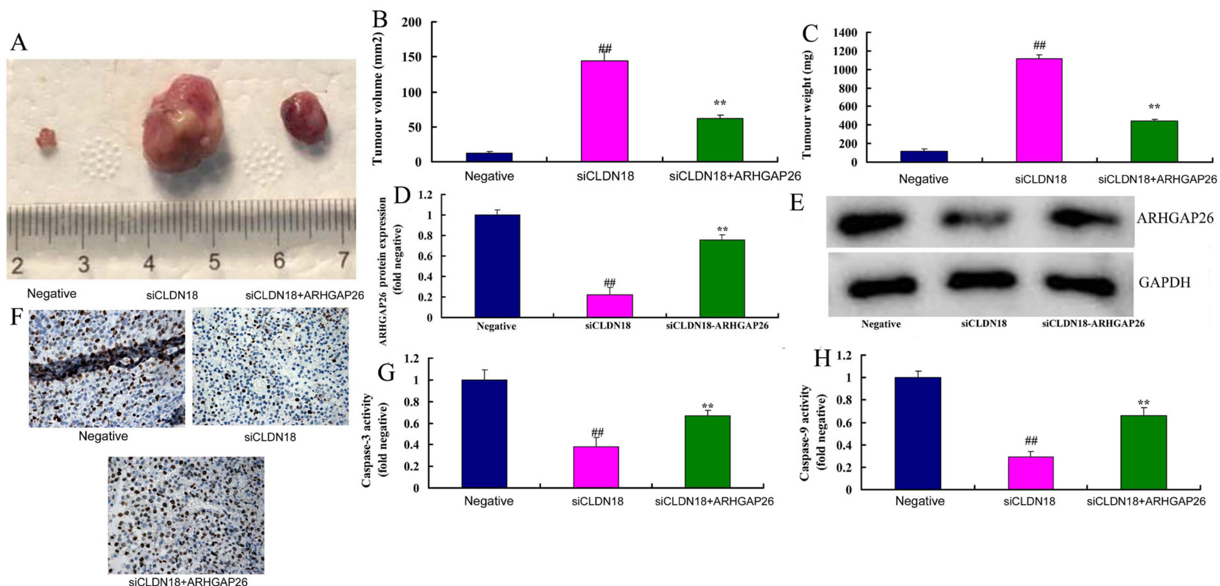

Figure 9. ARHGAP26 reduced the effects of CLDN18 downregulation in vivo model. Tumor volume and weight (A, B, C), ARHGAP26 protein expression (D, E), Immunohistochemical for ARHGAP26 (F), caspase-3/9 activity levels (G, H) in vivo model of gastric cancer. Negative, negative group; siCLDN18, down-regulation of CLDN18 group; siCLDN18+ARHGAP26, down-regulation of CLDN18 and over-expression of ARHGAP26 group. ##P < 0.05 vs negative group; **P < 0.05 vs down-regulation of CLDN18 group.

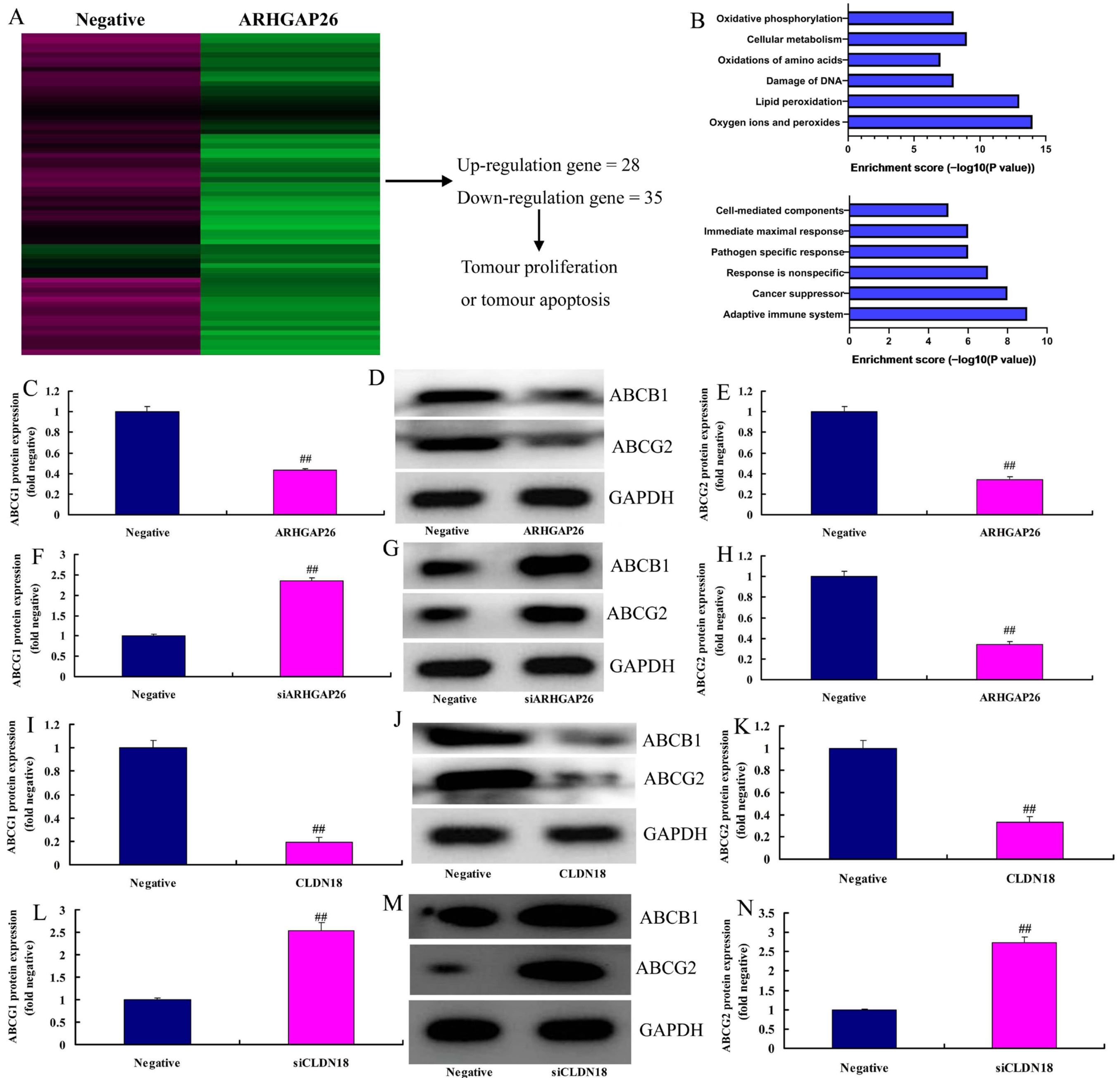

Figure 10. CLDN18-ARHGAP26 mediated tumor suppressive effects on gastric cancer cells by ABCG2 and ABCB1 pathway. Gene chip (A), network analysis graphics (B), ABCG2 and ABCB1 protein expression by over-expression of ARHGAP26 (C, D, E), ABCG2 and ABCB1 protein expression by down-regulation of ARHGAP26 (F, G, H), ABCG2 and ABCB1 protein expression by over-expression of CLDN18 (I, J, K), ABCG2 and ABCB1 protein expression by down-regulation of CLDN18 (L, M, N).

and vital signaling transduction pathway in cancer (Zhou et al., 2018). The activity of p53 is determined by CLDN18, and the second messengers generated by of CLDN18 regulate the serine-threonine protein kinase activity of p53 (Zhou et al., 2018). In this study, we found that Over-expression of CLDN18 promoted cell proliferation, cell migration and transferrer rate, and decreased apoptosis rate and caspase-3/9 activity levels *in vitro* model of gastric cancer. Luo et al. showed that CLDN18.1 reduced lung adenocarcinoma malignancy *in vivo* and *in vitro* (Luo et al., 2018). This might suggest a role for CLDN18 as a tumor suppressor of gastric cancer.

CLDN18 family members have been verified to be involved in cell apoptosis, with the majority of the family members functioning as anti-apoptosis (Sahin et al., 2008). CLDN18 regulates the apoptosis of vascular smooth muscle cell by modulating the transcription of p53 promotor (Sahin et al., 2008). However, CLDN18 reduced cell apoptosis and promoted cell proliferation via the p53 bypass system in vascular smooth muscle cells

(Hewitt et al., 2006). Recent studies have shown that CLDN18 can rapidly induce the expression of RHOA, followed by regulation of a series of expressions of "inflammation responsive genes", such as VEGF, TNF-α and MMP, in order to enhance the tolerance to inflammation responsive (Tan et al., 2019; Gon et al., 2005). Here we demonstrated that CLDN18 overexpression inhibits cell proliferation and induces cell apoptosis in gastric cancer cells by ARHGAP26. Coati et al. reported that CLDN18 is frequently expressed in gastric and gastro-oesophageal cancers (Sahin et al., 2008). This might suggest a role for CLDN18 as a tumor suppressor by dampening ARHGAP26 in gastric cancer.

ARHGAP26 protein is a proliferative indicator of cell, which reflects the relatively powerful proliferative ability of tumor cells (Katoh & Katoh, 2004). The cell proliferation is suppressed in gastric cancer with high expression of ARHGAP26 protein, which also harbors relatively potent invasion and malignant degree (Katoh & Katoh, 2004). ARHGAP26 is localized inside the cytomembrane and regulates cell proliferation, survival apoptosis at a translational level (Jarius et al., 2013). ARHGAP26 can reduced cell proliferation and induced cell apoptosis. CLDN18 plays a critical role in cell survival and apoptosis, with a very close relationship with tumor (Naskar et al., 2018). At present, in this study, these data showed that ARHGAP26 reduced the effects of CLDN18 downregulation *in vivo* model to suppressed RhoA and ROCK1 signaling pathway. Consequently, we speculate that CLDN18 modulates tumorigenesis linked to the ARHGAP26 and likely is associated with RhoA and ROCK1 signaling pathway in our study. Venerito et al. CLDN18-ARHGAP26/6 fusion was associated with signet-ring cell content from oxaliplatin/fluoropyrimidine-based chemotherapy (Venerito et al., 2019). In summary, CLDN18- ARHGAP26 suppressed ABCG2 and ABCB1 signaling pathway as a tumor suppressor in gastric cancer.

5 Conclusion

We provide the first evidence that CLDN18-ARHGAP26 expression was suppressed in patients with gastric cancer via ABCG2 and ABCB1 signaling pathway. Furthermore, expression levels of CLDN18 could provide prognostic information in patients with gastric cancer. Thus, establishment of standardized methods of CLDN18-ARHGAP26 quantification may be crucial for stratification of treatment modalities in patients with gastric cancer.

Conflict of interest

The authors declare that they have no competing interests.

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Author contributions

Jiachi Ma carried out the integrity of the entire study. Jing Li and Xuwei Zheng were involved in the study concepts, study design and definition of intellectual content. Jianguang Jia was dedicated to the literature research. Jing Li carried out the clinical studies, data analysis, statistical analysis and manuscript preparation. Jing Li, Xuwei Zheng, Bo Xie, Hu Wang and Chensong Zhang were involved in the experimental studies. Hongbo Li handled the data acquisition. Jing Li and Jiachi Ma were dedicated to the manuscript editing and manuscript review. All authors have read and approved this article.

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