# *COL11A1* is overexpressed in gastric cancer tissues and regulates proliferation, migration and invasion of HGC-27 gastric cancer cells *in vitro*

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**Abstract.** The role of *COL11A1* in carcinogenesis is increasingly recognized. However, the biological role and potential mechanisms of *COL11A1* in gastric cancer have not been elucidated. In the present study, the *COL11A1* mRNA expression in 57 patients with gastric cancer was measured by reverse transcription quantitative PCR (RT-qPCR), and the biological effects of *COL11A1* suppression were determined using MTS, monolayer colony formation, flow cytometry and Transwell assays. In addition, the potential molecular mechanisms of *COL11A1* in gastric cancer were analyzed by western blotting and cDNA microarray analysis. Compared with matched adjacent non-tumor tissue, *COL11A1* mRNA was significantly overexpressed in tumor tissue and was positively related to age, tumor invasion depth, tumor size and lymph node positivity. Moreover, *in vitro* experiments demonstrated that *COL11A1* suppression by short hairpin RNA (shRNA) significantly inhibited the proliferation, migration and invasion of HGC-27 cells and that *COL11A1* suppression promoted cell apoptosis, induced $G_1$-phase cell cycle arrest and led to a significant downregulation of cyclin $D_1$ and upregulation of p21 and cleaved caspase-3. In addition, the cDNA microarray analysis of HGC-27 cells with and without *COL11A1* suppression indicated that *COL11A1* may regulate multiple genes responsible for cell growth and/or invasion, including downregulation of *CDK6, TIAM1, ITGB8* and *WNT5A* and upregulation of *RGS2* and *NEFL* following suppression of *COL11A1* expression in HGC-27 cells, validated with RT-qPCR assays. Taken together, our findings demonstrate that *COL11A1* might be an oncogene in GC and is a promising therapeutic target in cancer treatment.

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## Introduction

Gastric cancer (GC) ranks the third cause of cancer mortality and the fifth most common cancer worldwide (1), while most patients with GC are diagnosed at the advanced stage which means a relatively poor prognosis of overall survival (2). As is well recognized, cancer results from accumulation of multiple molecular alterations in the same cells or their descendants (3). Hence, the identification of genes with oncogenic potential or tumor-suppressing activity may be of great use for diagnosis and treatment.

Previously, Zhao *et al* used an oligonucleotide microarray containing 38,500 genes in 11 patients with GC to distinguish malignant lesions from premalignant and normal ones, and type XI collagen α1 (*COL11A1*) gene expression was found upregulated in malignant tissue compared to premalignant tissue (4). As a member of minor fibrillar collagens, *COL11A1*, encoded by *COL11A1* gene located on chromosome 1p21, can be produced by cartilage and a variety of non-cartilaginous tissues, such as bone, vitreous, skin, and heart (5). Alterations of *COL11A1*, including at least four mRNA variants, can lead to several diseases such as Stickler syndrome type 2 (6), Marshall syndrome (7,8) and lumbar disc disease (9).

Besides, *COL11A1* has also been reported to be upregulated in various cancers, including colorectal, pancreatic, and ovarian cancer. Fischer *et al* first reported that *COL11A1* mRNA expression was significantly increased in colorectal cancer tissue compared with normal colonic tissue (10), and studies in pancreatic cancer (11), non-small cell lung cancer (12) and breast cancer (13) have also demonstrated increased levels of *COL11A1* in tumor tissue compared with normal tissue. Recent studies have implicated *COL11A1* in cancer cell growth and tumorigenicity. For example, *COL11A1* knockdown in head and neck squamous cell cancer gave rise to a reduction in cell growth and invasion *in vitro* (14), and *COL11A1* knockdown in ovarian cancer cells led to a decrease in cell proliferation and invasion *in vitro* and the metastasis of an ovarian tumor xenograft (15). Thus, *COL11A1* may represent a potential therapeutic target in need of further investigation.

Considering the limited studies regarding the relationship between *COL11A1* and GC, we evaluated the *COL11A1* mRNA expression in GC tissue, investigated the possible role of *COL11A1* in GC proliferation, cell cycle, apoptosis and invasion and explored the potential molecular mechanisms of *COL11A1* in GC cells in the present study.

## Materials and methods

*Ethics statement and clinical tissue samples*. This study was approved by the Clinical Research Ethics Committee of Sir Run Run Shaw Hospital of Zhejiang University (Hangzhou, Zhejiang, China) (permit number: 20110225-3200). Giving their informed consents to use their gastric tissue and publish their case details, all 57 consecutive patients who underwent surgery for GC in Sir Run Run Shaw Hospital were enrolled in this study. The histologic classification of gastric carcinoma is based on the 2010 WHO classification system and the clinicopathologic parameters including age, gender, tumor size, depth of invasion and lymph node positivity were obtained. The matched tumor tissues and adjacent non-tumor tissue samples were frozen immediately in liquid nitrogen and were stored at -80˚C until RNA extraction.

*Cell culture*. A total of four poorly differentiated (AGS, MKN-45, BGC-823 and MGC-803), one moderately differentiated (SGC-7901), one undifferentiated (HGC-27) and one well differentiated (MKN-28) gastric cancer cell line were included for the purposes of this study. These cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA) and Type Culture Collection of China Academic Science (Shanghai, China). One normal immortalized gastric epithelial cell line (GES-1) was obtained from the Beijing Institute for Cancer Research (Beijing, China). The cells were cultured in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Sijiqing, Huzhou, Zhejiang, China) and were incubated at 37˚C in 5% $CO_2$.

*Reverse transcription quantitative PCR (RT-qPCR) analysis*. Total RNA was extracted using TRIzol reagent (CW Biotech, Beijing, China), and the reverse transcription reaction was performed using 1 $\mu$g RNA with a reverse transcription kit (Takara, Otsu, Japan). RT-qPCR reactions were performed using a SYBR green PCR kit (Takara) in a LightCycler® 480 II Real-Time PCR System according to the manufacturer's instructions (Roche Diagnostics, Basel, Switzerland). U6 and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as endogenous controls for tissues and cells, respectively. The RT-qPCR conditions are as follows: one cycle of 95˚C for 30 sec, 40 cycles of 95˚C for 15 sec and 60˚C for 60 sec, melting curve analysis. The mRNA expression levels were determined using the $2^{-\Delta Ct}$ method for tissues and the $2^{-\Delta\Delta Ct}$ method for cells. The forward primer sequence is 5'-AGTGGCATCGGGTAGCAATCA-3' (located in exon 3-4, nt 806-826) and the reverse one is 5'-TGTCCC CCTCAAAAACTTCTTCAT-3' (located in exon 4-5, nt 953-976). The other primer sequences for the RT-qPCR assays are listed in Table I. All experiments were performed in triplicate.

Table I. The forward and reverse primer sequences used in experimental procedures.

| Gene name | Primer sequences |
|---|---|
| XIAP | F: GACAGTATGCAAGATGAGTCAAGTCA |
| | R: GCAAAGCTTCTCCTCTTGCAG |
| NEFL | F: AGCTGGAGGACAAGCAGAAC |
| | R: TGCCATTTCACTCTTTGTGG |
| RGS2 | F: GTTGGGTAGTGAATCAGGAAGC |
| | R: GACCACCTATTCCCTTCTTGC |
| ITGB8 | F: GGCCAAGGTGAAGACAATAGA |
| | R: ATCCTCTTGAACACACCATCC |
| WNT5A | F: ATCAATTCCGACATCGAAGG |
| | R: CGTTCACCACCCCTGCT |
| CDK6 | F: GTGCCCTGTCTCACCCATAC |
| | R: GACCCATAAGCCACCAAGG |
| TIAM1 | F: CAGGTGTTTGGAGAGGGAAC |
| | R: AATGTCGCAGTCAGGGTTG |
| U6 | F: CTCGCTTCGGCAGCACA |
| | R: AACGCTTCACGAATTTGCGT |
| GAPDH | F: GAAGGTGAAGGTCGGAGT |
| | R: GAAGATGGTGATGGGATTTC |

F, forward; R, reverse.

*Western blotting*. The cells were washed in cold PBS and lysed in RIPA lysis buffer (Beyotime, Jiangsu, China) on ice for 30 min. The cell lysates were centrifuged at 13,000 x g for 15 min at 4˚C, and the total protein concentration was analyzed with the BCA Protein Assay kit (Beyotime). Equal amounts of protein samples were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels (SDS-PAGE). The proteins were then transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% nonfat milk and incubated with primary antibodies at 4˚C overnight. The proteins were detected using chemiluminescence with a Las-4000 Imaging System (Fujifilm, Tokyo, Japan). The following primary antibodies were used: *COL11A1* (1:1000; Abcam, Cambridge, UK), pro-caspase-3 (1:1000; Cell Signaling Technology Inc., Danvers, MA, USA), cleaved caspase-3 (1:1000; Cell Signaling Technology Inc.), cyclin $D_1$ (1:1000; Cell Signaling Technology Inc.), p21 (1:1000; Cell Signaling Technology Inc.), p27 (1:1000; Cell Signaling Technology Inc.), cyclin-dependent kinase 2 (CDK2) (1:500; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), cyclin-dependent kinase 4 (CDK4) (1:500; Santa Cruz Biotechnology Inc.) and β-tubulin (1:1000; CW Biotech). All experiments were performed in triplicate.

*Plasmid transfection of stable cell lines*. The *COL11A1* short hairpin RNA (shRNA) plasmids (HSH002603-2-mH1, GeneCopoeia, Rockville, MD, USA) and *COL11A1* vector plasmids (CSHCTR001-mH1, GeneCopoeia) were purchased

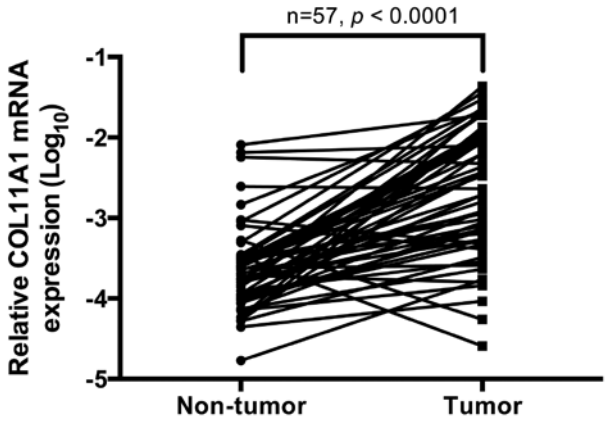


Figure 1. RT-qPCR analysis of *COL11A1* mRNA expression in GC patients. The relative expression of *COL11A1* mRNA was significantly increased in GC tissues compared with matched adjacent non-tumor tissues by RT-qPCR (n=57, p<0.0001).

and transfected into HGC-27 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) to establish stable cell lines. After 48 h, the transfectants were selected with 3 $\mu$g/ml puromycin (Amresco, Cleveland, OH, USA) for 2 weeks. A positive stably transfected clone was isolated and allowed to grow. Decreased *COL11A1* expression was confirmed by RT-qPCR and western blotting. All experiments were performed in triplicate.

*Cell proliferation assay*. A total of $3x10^3$ stably transfected cells in 100 $\mu$l medium were seeded into 96-well plates and incubated for 96 h. Then, 20 $\mu$l of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) (Promega, Madison, WI, USA) reagent was added to each well and incubated for 2 h. The absorbance values were measured by a multi-well plate reader (Molecular Devices, Sunnyvale, CA, USA) at 490 nm. All experiments were performed in triplicate.

*Colony formation assay*. A total of $6x10^2$ cells were plated into six-well plates in triplicate. The plates were incubated at 37˚C in 5% $CO_2$ for 10 days, and then the colonies were fixed with methanol and stained with crystal violet before counting. Values are presented as the mean percentage ± SD from three individual experiments, and the number for the *COL11A1* vector cells was set to 100%. All experiments were performed in triplicate.

*Cell migration and invasion assays*. Cell migration was assessed using Transwell migration assays (Corning, Inc., Corning, NY, USA). Briefly, $5x10^4$ cells were plated in the upper chamber in 200 $\mu$l medium containing 1% FBS. The lower chamber contained 600 $\mu$l medium containing 10% FBS. After 13 h of incubation, the non-migratory cells in the upper chamber were carefully removed with a cotton swab. The migrated cells were fixed with methanol and stained with DAPI. The cell numbers were counted in five random fields (x400 magnification) using a fluorescence microscope. All experiments were performed in triplicate.

Cell invasion was performed in Transwell chambers (Corning, Inc.) coated with BD Matrigel. For this assay, $1x10^5$ cells were plated in the upper chamber in 200 $\mu$l of medium containing 1% FBS. The lower chamber was filled with 600 $\mu$l of culture medium with 10% FBS. After 24 h, the membranes were fixed with methanol and stained with gentian violet. The cell numbers were counted by microscope in five random fields (x200 magnification). All experiments were performed in triplicate.

Table II. Clinicopathological characteristics and *COL11A1* mRNA expression in gastric cancer samples.

| Variables | Total | *COL11A1* expression ($2^{-\Delta Ct}$) (median) | P-value |
|---|---|---|---|
| Age (years) | | | |
| ≤63 | 27 (47%) | 0.00113 | 0.031[a] |
| ≥64 | 30 (53%) | 0.00548 | |
| Gender | | | |
| Male | 39 (68%) | 0.00193 | 0.352 |
| Female | 18 (32%) | 0.00356 | |
| Invasion depth | | | |
| $T_{2-4}$ | 44 (77%) | 0.00403 | 0.0004[b] |
| $T_1$ | 13 (23%) | 0.000409 | |
| Tumor size (cm) | | | |
| <5 | 27 (47%) | 0.000878 | 0.024[a] |
| ≥5 | 30 (53%) | 0.00630 | |
| Degree of differentiation | | | |
| Poorly | 37 (65%) | 0.00159 | 0.261 |
| Moderately/Well | 20 (35%) | 0.00630 | |
| Lymph node positivity | | | |
| Yes | 36 (63%) | 0.00455 | 0.019[a] |
| No | 21 (37%) | 0.000739 | |

[a]p<0.05, [b]p<0.01.

*Cell apoptosis and cell cycle analysis*. For cell apoptosis analysis, $2x10^5$ stable transfected cells were washed in cold PBS and suspended in 1X Annexin V Binding Buffer. Then, 5 $\mu$l FITC Annexin V (Becton Dickinson, San Jose, CA, USA) and 5 $\mu$l PI (Becton Dickinson) solutions were added. After incubation for 15 min, the stained cells were analyzed by flow cytometry on a FACScan analyzer (Becton Dickinson). All experiments were performed in triplicate.

For cell cycle analysis, $2x10^5$ stably transfected cells were harvested and washed in PBS. Cellular DNA was stained in the dark by a Cell Cycle Staining kit (Multisciences Biotech, Hangzhou, Zhejiang, China) for 30 min at room temperature. The cells were then analyzed by flow cytometry, and the cell cycle distribution was determined using ModFit LT software (Verity Software House, Topsham, ME, USA). All experiments were performed in triplicate.

*cDNA microarray analysis*. Total RNA was extracted from stably transfected HGC-27 cells with *COL11A1* shRNA and

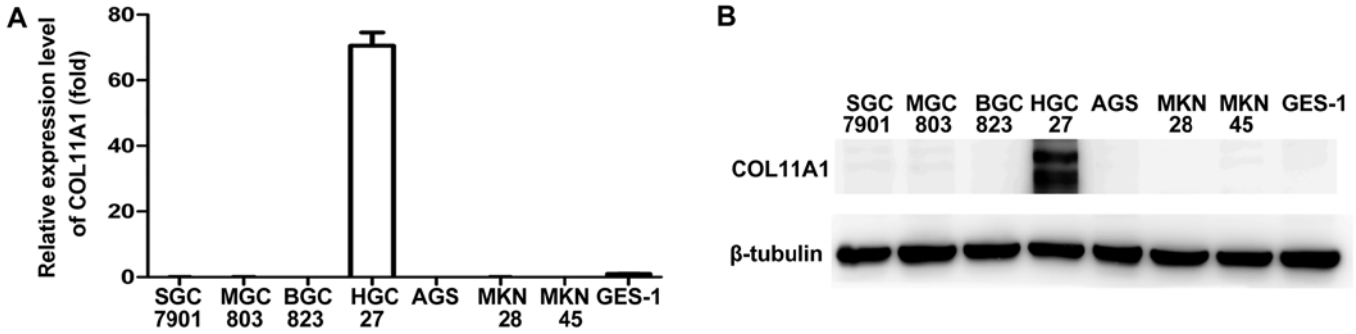


Figure 2. *COL11A1* expression in seven GC cell lines compared with normal immortalized epithelial cells (GES-1). (A) The *COL11A1* mRNA expression was higher in HGC-27 cells but lower in the other six GC cells (SGC-7901, MGC-803, BGC-823, AGS, MKN-28, and MKN-45) compared with GES-1 by RT-qPCR analysis. (B) *COL11A1* protein expression levels were higher in HGC-27 cells but lower in the other six GC cells (SGC-7901, MGC-803, BGC-823, AGS, MKN-28, and MKN-45) compared with GES-1 by western blotting.

Table III. The influences of cell apoptosis and cell cycle by *COL11A1* knockdown.

| Group | Early apoptotic cells $A_1$ (%) | Late apoptotic cells $A_2$ (%) | $G_1$ Phase (%) | S Phase (%) | $G_2$/M Phase (%) |
|---|---|---|---|---|---|
| COL11A1 vector | 7.42±0.89 | 11.19±0.25 | 28.57±0.24 | 50.50±0.48 | 20.93±0.25 |
| COL11A1 shRNA | 14.94±0.27[a] | 20.62±0.21[a] | 36.34±0.38[a] | 50.38±0.28 | 13.28±0.38[a] |

[a]p<0.01.

*COL11A1* vector. Total RNA was amplified, labeled and purified to obtain biotin-labeled cDNA. The labeled cDNA were hybridized to probes on Affymetrix U133 plus 2.0 arrays (Shanghai Biotechnology Corporation, Shanghai, China). The microarray data were analyzed using Gene Spring Software 11.0. We selected fold change (shRNA/vector) >3 or <0.333 as the threshold for upregulation or downregulation. The potential target genes were verified with RT-qPCR.

*Statistical analysis*. Wilcoxon matched pairs test was performed to compare paired data, Mann-Whitney U test was used to analyze and compare the medians of continuous variables, and Student's t-test was performed to compare two independent data. A value of p<0.05 was considered statistically significant. All data were analyzed using IBM SPSS Statistics 20.0.

## Results

We used RT-qPCR analysis to measure the *COL11A1* mRNA expression and found that the *COL11A1* mRNA expression level was significantly overexpressed in 57 GC tissues compared to matched adjacent non-tumor gastric tissue (p<0.0001, Fig. 1).

In 2/57 patients distant metastases occurred (liver and peritoneum, respectively), and 56/57 patients were diagnosed with gastric cancer of the adenocarcinoma subtype, while the remaining patient was diagnosed with an undifferentiated gastric carcinoma. The relationship between clinicopathological features and *COL11A1* expression in GC is shown in Table II. *COL11A1* mRNA expression was significantly positively related to age, tumor invasion depth, tumor size and lymph node positivity (p<0.05, p<0.01, p<0.05 and p<0.05, respectively), while there were no significant associations between *COL11A1* expression and patient gender (p>0.05) and degree of differentiation (p>0.05) in GC.

To determine which cell lines to use for further study, we compared *COL11A1* mRNA and protein expression levels in seven GC cell lines (SGC-7901, MGC-803, BGC-823, HGC-27, AGS, MKN-28, and MKN-45) with expression levels in normal immortalized epithelial cells (GES-1) by RT-qPCR and western blotting. The results showed that the relative *COL11A1* mRNA expression was higher in HGC-27 cells but lower in the other six GC cells (Fig. 2A), while similar results were observed by western blotting (Fig. 2B). Hence, HGC-27 was the only chosen GC cell line for further analysis.

To validate whether *COL11A1* contributed to GC proliferation, migration and invasion, we transfected HGC-27 cells with a plasmid encoding *COL11A1*-silencing shRNA (*COL11A1* shRNA) or *COL11A1* vector shRNA (*COL11A1* vector). The decreased *COL11A1* mRNA and protein expression levels were confirmed by RT-qPCR and western blotting (Fig. 3A).

Then, we examined whether *COL11A1* contributed to GC cell proliferation. The results of MTS assays showed significant cell growth inhibition in *COL11A1* shRNA HGC-27 cells compared with *COL11A1* vector cells (p<0.01 at 48, 72 and 96 h) (Fig. 3B). Consistently, the number of surviving colonies formed on the plates in *COL11A1* shRNA HGC-27 cells was also significantly reduced compared with *COL11A1* vector cells (p<0.01) (Fig. 3C). Thus, *COL11A1* contributed to cell proliferation in GC cells *in vitro*.

Next, Transwell migration and invasion assays were performed, and the results demonstrated that *COL11A1* knockdown in HGC-27 cells significantly suppressed migration (p<0.01) and invasion (p<0.01), respectively (Fig. 3D and E).

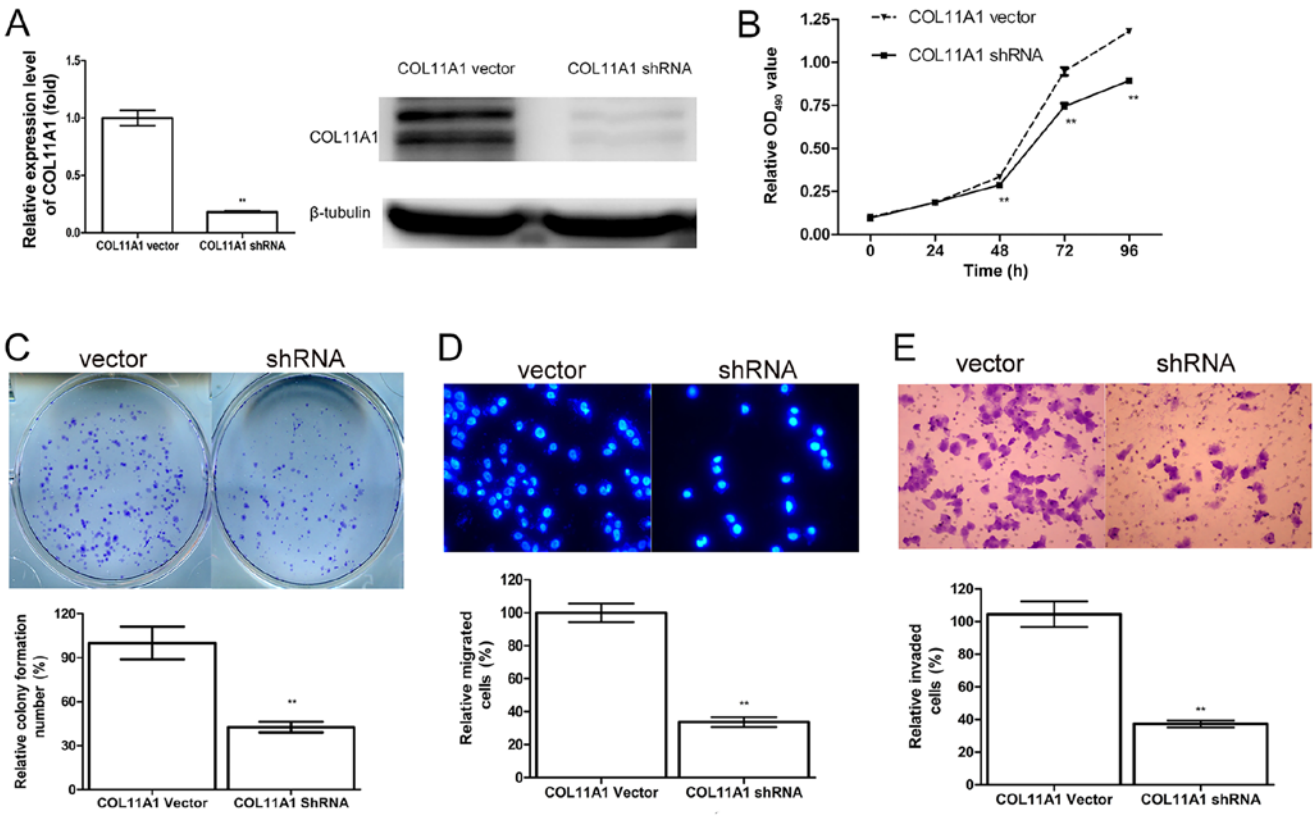


Figure 3. Suppressing *COL11A1* inhibits the proliferation, migration and invasion of HGC-27 cells *in vitro*. (A) Decreased *COL11A1* expression in HGC-27 GC cells was confirmed by RT-qPCR (left) and western blotting (right). (B) The MTS assays showed that *COL11A1* suppression significantly inhibited proliferation. (C) The monolayer colony formation assays showed that *COL11A1* suppression significantly inhibited proliferation. (D) Transwell migration assays of *COL11A1* downregulated HGC-27 cells showed that silencing *COL11A1* significantly inhibited cell migration. (E) Transwell invasion assays of *COL11A1* downregulated HGC-27 cells showed that suppressing *COL11A1* significantly inhibited cell invasion. The asterisk indicates statistical significance ($^{*}p<0.05$, $^{**}p<0.01$).

Cumulatively, these results indicated that *COL11A1* played a role in cell proliferation, migration and invasion of HGC-27 GC cells *in vitro*.

To explore the underlying mechanisms of the growth inhibition by *COL11A1* knockdown, we assessed cell apoptosis and cell cycle by flow cytometry. *COL11A1* suppression significantly induced apoptosis compared with *COL11A1* vector (Fig. 4A and Table III), and a significant accumulation of cells in $G_1$ phase was observed in *COL11A1* knockdown cells compared to that in *COL11A1* vector cells (Fig. 4B and Table III). Then, we further evaluated expression levels of several cell cycle and apoptosis-related proteins, and we found that *COL11A1* suppression led to upregulation of the cell cycle inhibitor p21 but not p27 and reduced cyclin $D_1$ but not CDK2 and CDK4. We also found activation of the apoptotic protein caspase-3 (Fig. 4C).

cDNA microarray in HGC-27 cells with and without *COL11A1* knockdown was performed to identify potential downstream genes of *COL11A1* in GC, and the results indicated that *COL11A1* suppression altered the expression of multiple genes involved in cell proliferation and invasion (Fig. 5A). The results of RT-qPCR further confirmed that *CDK6, TIAM1, XIAP, ITGB8* and *WNT5A* were downregulated, and *RGS2* and *NEFL* were upregulated by *COL11A1* suppression in HGC-27 cells (Fig. 5B). These results suggested that *COL11A1* may play a role in tumor development and progression through regulation of these genes.

## Discussion

The gradual accumulation of genetic alternations contributes to the development and progression of cancers. In the present study, we observed that overexpression of *COL11A1* mRNA was related to tumor age, tumor size, depth of invasion and lymph node positivity, and that *COL11A1* could regulate cell proliferation, migration and invasion through several potential downstream genes.

Several lines of evidence indicate that *COL11A1* expression is upregulated in various cancers such as ovarian (15), colorectal (10), breast (13), pancreatic (16) and head and neck squamous cell (14) cancers, suggesting an oncogenic role of *COL11A1* in carcinogenesis. However, the role of *COL11A1* in GC has not been elucidated. This study demonstrated that *COL11A1* mRNA was overexpressed in 57 GC tissues compared with matched non-tumor tissues by RT-qPCR analysis, which is consistent with previous studies (4). In addition, we analyzed the relationship between *COL11A1* mRNA expression and clinicopathological characteristics. Consistent with a previous report in GC by Affymetrix analysis (17), *COL11A1* mRNA expression in the advanced GC was significantly higher than that in the early GC. Besides, high *COL11A1* expression was positively related to tumor age, tumor size and lymph node positivity, which indicates *COL11A1* may be involved in GC growth and invasion. These results supports that *COL11A1* may play a role in GC proliferation and invasion.

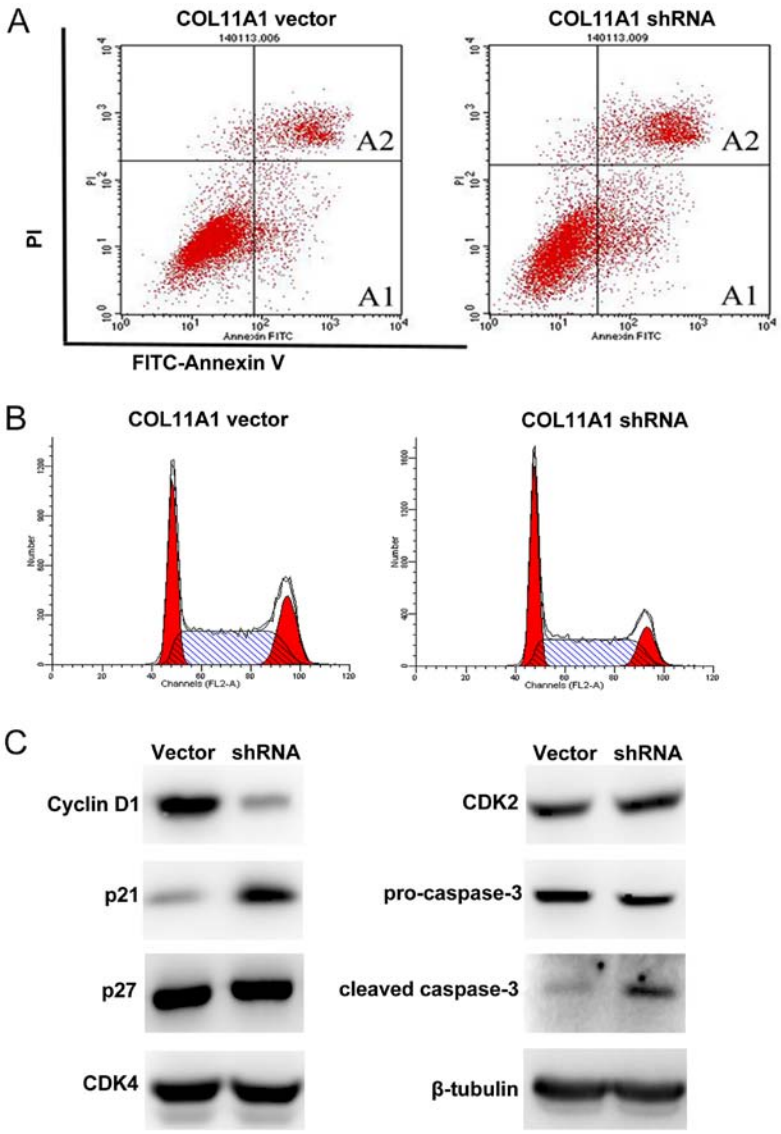


Figure 4. *COL11A1* suppression induces cell apoptosis and cell cycle inhibition in HGC-27 cells. (A) The rate of cell apoptosis was increased in cells with *COL11A1* suppression. Region $A_1$ indicates early apoptotic cells, while region $A_2$ indicates late apoptotic cells. (B) Suppression of *COL11A1* significantly increased the proportion of cells at $G_1$ phase from 28.57±0.24% to 36.34±0.38%. (C) *COL11A1* suppression arrested cells at $G_1$ phase of the cell cycle by downregulating protein expression of cyclin $D_1$ and upregulating protein expression of p21, while induced apoptosis by upregulating protein expression of cleaved caspase-3. Band densities were normalized to β-tubulin expression.

To study the biological effects of *COL11A1* in GC, we examined *COL11A1* expression in seven GC cell lines and one GES-1 cell line and found that HGC-27 was the only cell line showing *COL11A1* upregulation compared to GES-1. The reason why *COL11A1* is overexpressed in only one cancer cell line, out of seven, compared to normal cells needs further explanation. From our perspective, one explanation may be that HGC-27, a kind of undifferentiated and relatively less attached cell, is much more aggressive than the other six cells. Finally, we chose HGC-27 for the next study.

Based on depletion experiments *in vitro*, we found that silencing of *COL11A1* significantly decreased the proliferation, migration and invasion of HGC-27 cells, which is consistent with two previous reports in ovarian cancer (15) and head and neck squamous cell cancer (14). Furthermore, we studied the cell cycle and apoptosis using flow cytometry, and the results showed that *COL11A1* suppression significantly induced cell cycle arrest at $G_1$ phase and promoted cell apoptosis. $G_1$/S phase transition is a major checkpoint for cell cycle progression. Cyclin $D_1$, forming functional kinase complexes with CDK4 or CDK6, is a periodic regulatory protein that governs cell cycle transit from $G_1$ phase into S phase and is abnormally expressed in many human cancers (18,19). There are also inhibitory proteins preventing the cell cycle. Among these inhibitors, p21, a potent cyclin-dependent kinase inhibitor binds and inhibits the activity of cyclin $D_1$-CDK4/6 complexes controlling the transition from $G_1$ to S phase (20). In the present study, western blotting demonstrated that suppression of *COL11A1* decreased cyclin $D_1$ expression and increased p21 expression. However, there was no significant effect on CDK2, CDK4 or p27. These results suggest that $G_1$/S cell cycle arrest induced by *COL11A1* suppression is mediated through the p21 and cyclin $D_1$ pathway. In addition, caspase-3 is a well-recognized indicator of cellular apoptosis (21) and is activated in apoptotic cells by both the extrinsic and intrinsic pathways (22). Our data indicated that *COL11A1* suppression increased cleaved caspase-3 levels in

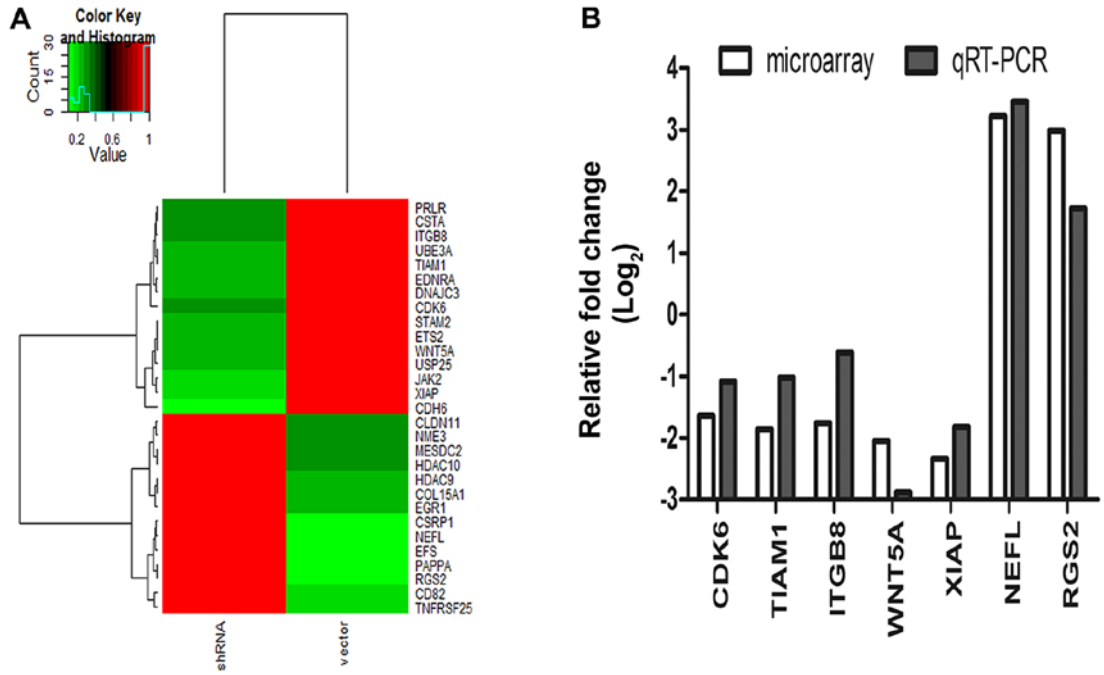


Figure 5. Gene expression changes by *COL11A1* suppression in HGC-27 cells. (A) The representative gene expression profiles in HGC-27 cells with stably transfected *COL11A1* shRNA compared with *COL11A1* vector are indicated on the right side of this heatmap image. Green indicates small values, progressing to red for higher values. (B) The expression of nine potential target genes was verified by RT-qPCR. $Log_2$ fold change of relative expression levels in *COL11A1* shRNA HGC-27 cells were compared with that in *COL11A1* vector, which was normalized to 1. □, RT-qPCR data; ■, results of cDNA microarray.

HGC-27 cells, and thus promoted apoptosis of HGC-27 cells. Further studies are required to determine whether the extrinsic or intrinsic pathway is involved. Despite several attempts, we failed to establish a successful nude mouse model to investigate the role of *COL11A1 in vivo*.

The molecular mechanisms of *COL11A1* action in cancers remain unclear, and the only pathway studied is the *COL11A1-TGF-β1-MMP3* axis through which *COL11A1* promotes ovarian cancer aggressiveness. Therefore, we studied the molecular mechanisms of *COL11A1* in GC by cDNA microarray. Numerous genes were altered in cells with *COL11A1* knockdown compared to cells with *COL11A1* vector, and the representative potential target genes have been previously reported to participate in cell growth, migration and invasion. As a member of the inhibitor of apoptosis protein gene family, *XIAP* can inhibit caspases and suppress apoptosis, and a previous study showed that downregulation of *XIAP* induced apoptosis was related to activation of caspase-3 in GC (23). *CDK6*, important for the $G_1$ phase progression and $G_1$/S transition, was upregulated in many cancers (24). Another *COL11A1* potential target gene, *RGS2*, was also involved in cell growth in breast cancer (25) and prostate cancer (26). In addition to the regulation of cell proliferation pathways, *COL11A1* downregulation decreased *TIAM1* which was involved in cell invasion in retinoblastoma (27) and gastric cancer (28). Furthermore, *NEFL*, a putative tumor suppressor gene, can inhibit cell proliferation and invasion in head and neck squamous cell carcinoma (29) and neuroblastoma (30). We further validated that *CDK6, TIAM1, XIAP, ITGB8* and *WNT5A* were downregulated, while *RGS2* and *NEFL* were upregulated in HGC-27 cells with *COL11A1* suppression using RT-qPCR analysis. Thus, the identification of potential target genes of *COL11A1* supported the hypothesis that *COL11A1* may modulate potential downstream genes to regulate cell proliferation and invasion in GC.

In conclusion, our study indicates that *COL11A1* may play an oncogenic role in the proliferation, migration and invasion in gastric cancer and may be a promising therapeutic target in cancer treatment. Further study is needed to clarify the potential specific molecular mechanisms of *COL11A1* in gastric cancer.

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