



Snail-induced epithelial-mesenchymal transition in gastric carcinoma cells and generation of cancer stem cell characteristics

Y.J. Yang, Z.B. Li, G.R. Zhang, L.J. Wu, J.Y. Yu, L.J. Hu, Y.L. Zhou, H.D. Wang and D. Liang

Hainan Province Nongken Sanya Hospital, Sanya, China

Corresponding author: D. Liang

E-mail: liangdongnsh@126.com / liangdong-1224@163.com

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ABSTRACT. Biological changes in Snail-overexpressed SGC7901 cells were studied by establishing a pEGFP-C1-Snail carrier. The significance of Snail in epithelial-mesenchymal transition (EMT) as well as the invasion and metastatic capacity of gastric cancer cells was also discussed; moreover, we attempted to verify the probable cancer stem cell characteristics of Snail-overexpressed cells. A pEGFP-C1-Snail eukaryotic expression plasmid was constructed and pEGFP-C1(-) and pEGFP-C1-Snail plasmids were extracted and transfected into SGC7901 cells using Lipofectamine 2000. Stably expressed SGC7901-N [control group containing pEGFP-C1(-)] and SGC7901-S (test group containing pEGFP-C1-Snail) cells were screened using a G418 resistance medium. Snail, E-cadherin, β -catenin, vimentin, and fibronectin gene and protein expressions were detected by real-time quantitative PCR, western blot, and immunofluorescence. Cell invasion and metastasis were tested by scratch test, invasion assay, and an

adhesion experiment. The positive rate of aldehyde dehydrogenase-1 (ALDH-1) expression was analyzed by flow cytometry. The results indicated the occurrence of EMT, accompanied by morphological changes in the cells and a weakening of the cell adhesion capacity. We also observed a decrease in the expression of epithelial markers E-cadherin and β -catenin and an increase in mesenchymal (Snail and vimentin) marker expression. Moreover, the cells showed increased invasiveness and metastatic capacity, and decreased proliferative ability. Moreover, the Snail-treated SGC7901 cells moved towards the scratch and produced fewer clones compared to the control cells. Owing to its capacity for self-renewal, SGC7901-S cells produced new clones and expressed ALDH-1. Therefore, we concluded that *Snail* overexpression induced EMT and endowed cells with tumor stem cell characteristics.

Key words: Epithelial-mesenchymal transition; Gastric carcinoma; Cancer stem cell; Snail

INTRODUCTION

Stomach cancer is the fourth most common type of tumor and has the second highest mortality rate worldwide (Torre et al., 2015) because of its invasiveness and metastatic capacity (Jemal et al., 2004; Iwahashi et al., 2016). Pathogenesis-oriented intervention is the precursor for therapeutic breakthroughs in stomach cancer. Epithelial-mesenchymal transition (EMT), the depolarization of epithelial cells into transferable mesenchymal cells, is the key cause of tumor formation. Cancer stem cells (CSCs), the source of tumor onset and development, have the capacity of infinite self-renewal and produce heterogeneous cancer cells in tumor tissues (Clarke et al., 2006). Therefore, EMT and CSCs must be thoroughly investigated to develop therapeutic strategies against stomach cancer. EMT plays an important role in the generation of CSCs and affects tumor invasiveness and metastasis; that is, EMT endows tumor cells with the characteristics of stem cells (thereby increasing the expression of CSCs), in addition to migratory and invasive capacities (Sohal, 2016). The transcription factor Snail is highly expressed in breast cancer (Ganesan et al., 2016), stomach cancer (Rosivatz et al., 2002), liver cancer (Nart et al., 2010), and colon cancer (Pálmer et al., 2004) cells. Snail is closely associated with histological differentiation, as well as lymphatic metastasis, in breast cancer. High Snail expression is also one of the major influencing factors of EMT in tumor cells (Cano et al., 2000; Moirangthem et al., 2016). Therefore, the aim of this study was to explore the significance of Snail overexpression in EMT in gastric carcinoma cells; additionally, we analyzed its effect on tumor invasiveness and metastasis at the molecular level, and attempted to verify the effect of Snail overexpression on the introduction of CSC characteristics to stomach cancer cells. The results of this study provide valuable evidence for stomach cancer treatment in clinical practice.

MATERIAL AND METHODS

Cell culture

SGC7901 cells purchased from the Shanghai Institute for Biological Sciences at the

Chinese Academy of Sciences were cultured in RPMI 1640 nutrient solution supplemented with 10% fetal calf serum at 37°C and saturation humidity in a 5% CO₂ incubator; SGC7901 cells in the logarithmic phase digested with 0.25% pancreatin-EDTA.

Construction of plus sense total-length Snail eukaryotic expression vector

*Hin*DIII and *Bam*HI were introduced to the primers (underlined in the primer sequence) designed according to the *Snail* gene sequence (GenBank ID: NM_005985), and total-length primer expression was designed for PCR amplification. Total RNA was extracted from SGC7901 cells using a one-step Trizol (Invitrogen, Carlsbad, CA, USA) method; RNA was reverse transcribed to cDNA and amplified by PCR under the following conditions: denaturation at 95°C for 2 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 63°C for 30 s, and extension at 72°C for 1 min, and a final extension at 72°C for 10 min. The PCR product was tested by 1% agarose gel electrophoresis (AGE) and purified using a commercial PCR purification kit (Qiagen, Venlo, Netherlands). The PCR product was connected to the cohesive end of a double-digested pEGFP-C1 vector (Shanghai Dingguo Biological Technology Co., Ltd., China) for transformation and subsequently, sequencing; the plus-sense pEGFP-C1-Snail expression vector was finally obtained. The total-length Snail expression primer sequences were as follows: upstream, 5'-CCC AAG CTT AAT GCC GCG CTC-3'; downstream, 5'-CGG GAT CCT CAG CGG GGA CAT C-3'.

Lipofection

The SGC7901 cells were transfected with the pEGFP-C1(-) and pEGFP-C1-Snail vectors using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer protocols. Highly expressed and stable positive clones were screened by culturing in G418 medium (Invitrogen). The cells were grouped based on the type of added vector: wild-type group (SGC7901 cells treated with Lipofectamine™ 2000 in the absence of plasmid DNA), SGC7901-S (treated with pEGFP-C1-Snail), and SGC7901-N [transfected with pEGFP-C1(-)].

RT-PCR

Cells in the logarithmic phase of growth were homogenized with the Trizol reagent to extract the total RNA. cDNA was synthesized using a reverse transcription kit (Life Technologies, MD, USA), according to the manufacturer instructions. The products of the reverse transcription reaction (2 μL) were amplified by PCR following the conditions: denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s, and a final extension at 72°C for 10 min. GAPDH was used as the internal reference. The primers were designed by Life Technologies. The sequences of all primers used are as follows: Snail: upstream, 5'-TTCTTCTGCGCTACTGCTGCG-3' and downstream, 5'-GGGCAGGTATGGAGAGGAAGA-3'; E-cadherin: upstream, 5'-GGATGTGCTGGATGTGAATG-3' and downstream, 5'-AGCAAGAGCAGCAGAATCAG-3'; β-catenin: upstream, 5'-GCTGAAGGTGCTATCTGTCTGCTC-3' and downstream, 5'-TGAACAAGACGTTGACTTGGATCTG-3'; vimentin: upstream, 5'-TGAGTACCGGAGACAGGTGCAG-3' and downstream, 5'-TAGCAGCTTCAACGGCAAAGTTC-3'; fibronectin: upstream,

5'-TGCCTTGCACGATGATATGGA-3' and downstream 5'-CTTGTGGGTGTGACCTGAGTGAA-3'; and GAPDH: upstream, 5'-GATGAGATTGGCATGGCTTT-3' and downstream 5'-CACCTTCACCGTTCCAGTTT-3'.

The PCR products were renatured at 54°C, analyzed by 10 g/L AGE, and the gel photographed. Gray value of the electrophoretic bands was scanned using a gel image analyzer (Mediacybernetics, MD, USA). Semi-quantitative mRNA levels are reported as the ratio of Snail, E-cadherin, β -catenin, vimentin, fibronectin, and GAPDH.

Western blot analysis of protein expressions

Total protein was extracted from SGC7901, SGC7901-S, and SGC7901-N cells by Coomassie brilliant blue semi-quantitative analysis. The proteins (50 μ g) were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride (PVDF) film, and blocked with 5% skimmed milk at 37°C for 1 h. The film was then incubated with antibodies against β -actin (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), E-cadherin (1:1000; Cell Signaling Technology, Danvers, MA, USA), vimentin (1:500; Santa Cruz Biotechnology), fibronectin (1:500; Santa Cruz Biotechnology), and Snail (1:500; Abcam, Cambridge, UK) overnight at 4°C. The film was then washed thrice with TBST (5 min each), incubated with the secondary antibody IgG (1:1000) tagged with alkaline phosphatase at 37°C for 1 h and subsequently rinsed with TBST for 10 min. Color development was achieved by adding the substrate (NBT/BCIP). The protein bands were subjected to a gray-scale analysis using the AlphaEase FC image analysis software, with β -actin as the internal reference (i.e., the gray scale of the target protein was determined relative to that of β -actin). The experiment was repeated thrice.

Immunofluorescence

The cells were cultured on a cover slip, fixed with 4% paraformaldehyde (PFA) for 20 min at 21°C and treated with 1% Triton X-100 for 15 min. The cells were then blocked with goat serum albumin at 37°C for 30 min and incubated with antibodies against E-cadherin (1:100; Cell Signaling Technology), vimentin (1:100; Santa Cruz Biotechnology), and fibronectin (1:100; Santa Cruz Biotechnology) at 4°C overnight. The cell nuclei were then incubated with the secondary antibody (1:50) and DAPI (1:1000; Invitrogen) for 2 min each. The immunofluorescent staining was observed using a Zeiss LSM-710 laser scanning confocal microscope (Carl Zeiss AG, Oberkochen, Germany).

Cell adhesion testing

The cells were inoculated on a 6-well plate (Corning, Corning, NY, USA) at a density of 4×10^5 cells/well; the cells were trypsinized after 1-2 h, and the adherent cells were calculated using an inverted microscope (Olympus, Tokyo, Japan). Three different adherent rates of the cell population were subsequently calculated.

Cell invasion assay

The extracellular matrix (ECM) gel (Sigma-Aldrich, St. Louis, MO, USA) was diluted

with dimethyl sulfoxide (DMSO; 1:1), and evenly coated onto the bottom of a Transwell loculus (well diameter: 8 μm) (Costar, Lowell, MA, USA) gel pavement without bubbles (approximately 20 μL per well; 0.5 mm thick). The well was incubated at 37°C for 30 min or until the gel solidified. Simultaneously, SGC7901-S and SGC7901-N at a confluence and adherence of 75% were digested, centrifuged, rinsed twice with phosphate-buffered saline (PBS), and counted. The cell density was adjusted to 1×10^5 cells/mL using a serum-free medium. Two hundred microliter of the cell suspension was added to the upper loculus and 500 μL medium supplemented with 10% fetal bovine serum (FBS) was added to the lower loculus. The Transwell loculus was then incubated at 21°C for 24 h. Subsequently, the Transwell loculus was rinsed twice with PBS, fixed with 4% PFA for 30 min, rinsed again with PBS (twice), and stained with crystal violet (Beyotime Institute of Biotechnology, Jiangsu, China) for 30 min. The cells were then rinsed twice with deionized water, and the Matrigel and cells in the upper loculus were gently wiped off using a cotton swab. The cells were photographed under a microscope, and the number of transmembrane cells were randomly calculated using the 10-vision option, for statistical analysis.

Scratch test

SGC7901-S and SGC7901-N cells were cultured overnight in serum-free medium on a 6-well plate (Corning). The medium was then rendered with a 0.5-cm long scratch. The cells were digested with trypsin, and the cell density adjusted to 5×10^5 cells/mL. The cells were re-inoculated onto the 6-well plate and incubated overnight. Even, thick straight lines were scratched perpendicular to the original scratch using a 10- μL pipette tip. The plate was rinsed twice with PBS to remove cells at the edges of the scratch, and serum-free medium was added for routine culture. These cells were photographed under a microscope 0, 12, 24, and 48 h after rendering the scratch, and the cell changes were recorded.

Flow cytometry

The samples were prepared using the Aldefluor kit (Stemcell Technologies, Vancouver, Canada), and analyzed per the manufacturer instructions. A single-cell suspension was prepared, the cells were counted, and 4×10^5 cells were centrifuged. The supernatant was discarded and the cells were resuspended in reaction buffer (400 μL) at a cell density of 1×10^6 cells/mL. The cell suspension (200 μL) was collected in a test tube, and 2 μL activated Aldefluor substrate was added to this and thoroughly mixed; the control tube contained 2 μL DEAB, to which 200 μL cell suspension was immediately added and mixed thoroughly. The tubes were incubated at 37°C for 30 min; subsequently, the tubes were centrifuged for 5 min to remove the supernatant. The cells were resuspended in 200 μL buffer in an ice-bath in the dark. The samples were then analyzed using a flow cytometer.

Statistical analysis

All data are reported as means \pm standard deviation (SD) of three or more independent tests. The statistical significance of obtained results was evaluated by one-way analysis of variance (ANOVA) and an unpaired *t*-test. P values <0.085 were considered statistically significant.

RESULTS

Construction of pEGFP-C1-Snail vector

The pEGFP-C1-Snail recombinant plasmid, connecting Snail to the cohesive end of the double-digested pEGFP-C1 vector (Figure 1A) by *Hin*DIII and *Bam*HI, contained an 800-bp specific Snail target fragment and a 4.7-kb vector fragment (Figure 1B). Sequencing by Shangahi Yingjun Co., Ltd., and subsequent comparison confirmed the sequence as a complete matching Snail sequence.

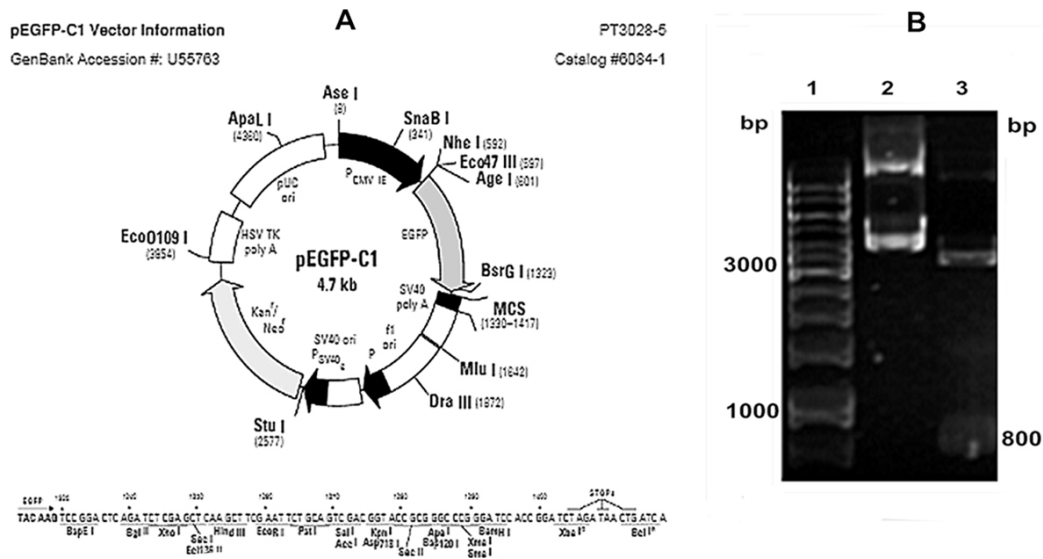


Figure 1. A. pEGFP-C1 vector; B. analysis of digested pEGFP-C1-Snail recombinant plasmid: lane 1 = marker; lane 2 = pEGFP-C1-Snail; lane 3 = cut.

Overexpression of Snail induces EMT in SGC7901 cells and alters the cell phenotype

Snail induces EMT and depolarization of epithelial cells, thereby granting the cells with the characteristics of mesenchymal cells. In this study, the eukaryon plasmids pEGFP-C1(-) and pEGFP-C1-Snail were transfected into SGC7901 cells. Positive clones were screened using G418 medium; monoclones were selected for further culture. Microscopic observation of stably expressed SGC7901-S, SGC7901-N, and wild-type cells acquired after 6 weeks showed evident fibrosis in SGC7901-S cells (elongated spindle cells with loose structure); however, SGC7901-N and wild-type cells retained an epithelioid morphology with dense paving stone structures (Figure 2).

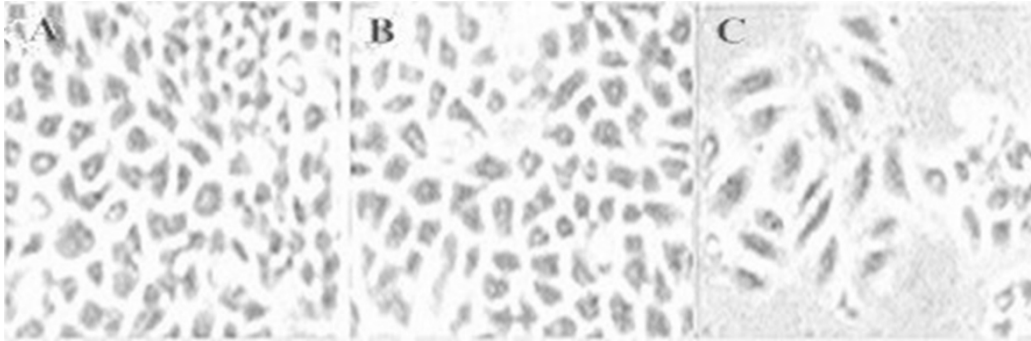


Figure 2. Overexpression of Snail induced morphological changes in SGC7901 cells. Wild-type (A) and SGC7901-N (B) cells present typical epithelioid morphology with dense paving stone structures, whereas SGC7901-S (C) cells exhibit evident fibrosis with loose structure.

Real-time quantitative PCR and western blot

Total RNA and total protein were extracted from two groups of cells. Real-time RT-PCR showed that Snail expression in SGC7901-S cells was 1.64 and 1.78 times higher than that in SGC7901-N and wild-type cells, respectively, confirming successful transfection. Snail overexpression led to a decrease in the expression of epithelial markers E-cadherin and β -catenin in SGC7901-S cells, compared to that in SGC7901-N and wild-type cells; however, we observed an increase in the expression of the mesenchymal markers vimentin and fibronectin in the former (Figure 3A). These results were verified by western blotting, wherein we observed a similar change in the expression of epithelial and mesenchymal marker proteins (downregulation of E-cadherin and β -catenin and upregulation of vimentin and fibronectin in SGC7901-S cells) (Figure 3B).

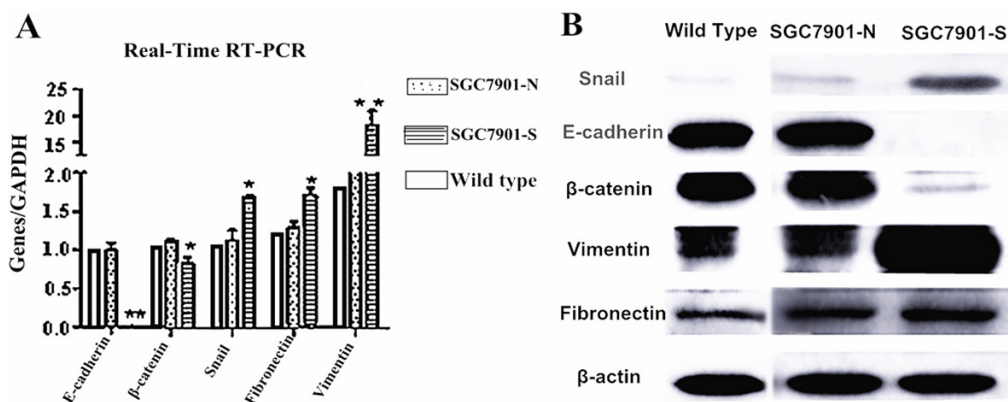


Figure 3. Results of real-time quantitative PCR (A) and western blot analysis (B) of E-cadherin, β -catenin, Snail, vimentin, and fibronectin gene and protein expressions (taking GAPDH and β -actin as the internal references for each analysis, respectively).

LSCM analysis presented evident morphological changes (long fibroblasts) as well as increased Snail and fibronectin (orange) expression and decreased E-cadherin (red) expression in SGC7901-S cells. However, SGC7901-N cells retained their epithelial morphology and showed normal E-cadherin and fibronectin expression, similar to the SGC7901 parent cells (Figure 4A and B). Therefore, increased expression of Snail in SGC7901 cells induced EMT.

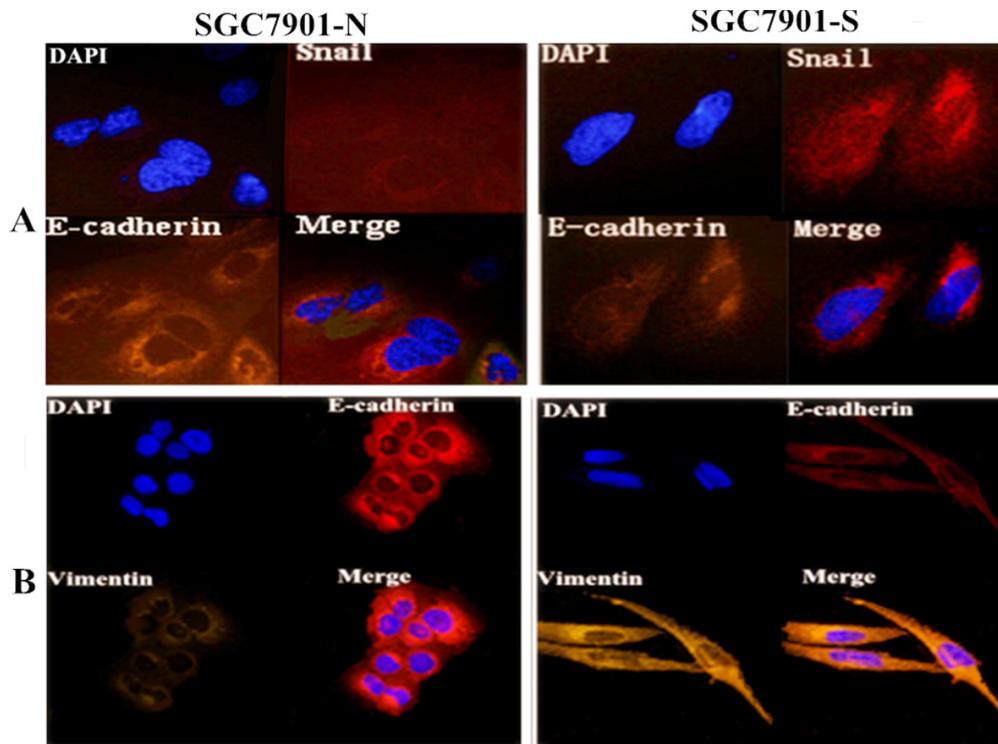


Figure 4. Immunofluorescence results of SGC7901-S and SGC7901-N cells. **A.** E-cadherin (orange), Snail (red); **B.** E-cadherin (red), vimentin (orange).

Overexpression of Snail in SGC7901 cells changed biological characteristics

As evidenced by the cell adhesion experiments, the SGC7901-S cells displayed a lower adhesive capacity compared to wild-type and SGC7901-N cells (Figure 5A). The rate of adhesion of wild-type, SGC7901-N, and SGC7901-S cells increased from 18.79, 16.01, and 12.69% at 1 h post-adhesion, to 49.36, 50.12, and 30.59% at 2 h post-adhesion, respectively. The invasive capacity of wild-type, SGC7901-N, and SGC7901-S cells was evaluated by the Transwell test. The substrate membrane-penetrating ability of cells after 24 h was analyzed by randomly selecting 10-vision (x100 amplification) to calculate the number of transmembrane cells (required to statistically analyze the invasive capacity of the three groups of cells). Quantitative analysis revealed the number of transmembrane cells in SGC7901-S cells to be 2.78 and 3.18 times higher than that in the wild-type and SGC7901-N cells, respectively (Figure 5B). Moreover, SGC7901-S cells showed higher invasiveness compared

to wild-type and SGC7901-N cells (Figure 5C). Therefore, Snail overexpression weakened the adhesiveness, but enhanced the invasiveness, of SGC7901 cells. These findings further confirmed the occurrence of EMT in SGC7901 cells.

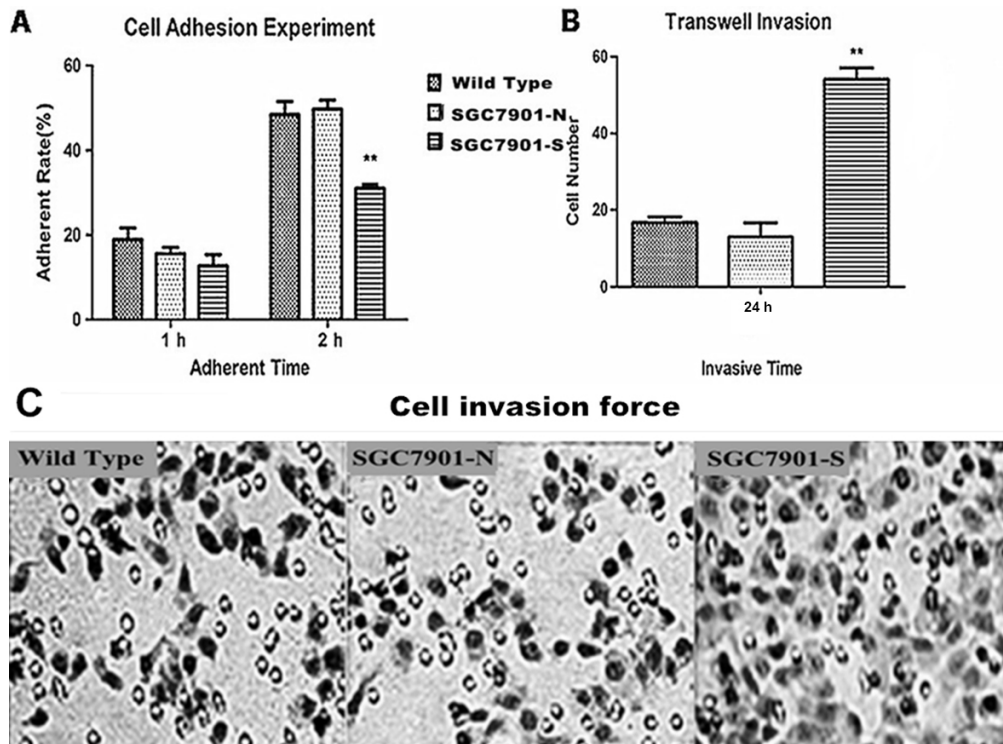


Figure 5. Cell adhesion (A), transwell invasion (B), and cell invasion assay (C).

Snail overexpression in SGC7901 cells induced CSC characteristics in SGC7901 cells

Wild-type and SGC7901-N cells were subjected to convergence 48 h after inflicting the scratch. However, the SGC7901-S cells failed to heal the wound (Figure 6). Accordingly, SGC7901-S cells with strong invasiveness failed to grow well and displayed some characteristics of CSCs. In other words, Snail overexpression in SGC7901 cells could have endowed the cells with characteristics of CSCs, therein leading to EMT and lowering the proliferation capacity.

The Aldefou kit was employed to stain the SGC7901-N and SGC7901-S cells. The FCM test results showed that the rates of positive expression of aldehyde dehydrogenase-1 (ALDH-1) in SGC7901-N and SGC7901-S cells were 1.05 and 8.37%, respectively, indicating that the SGC7901-S group contained a greater number of CSCs (Figure 7).

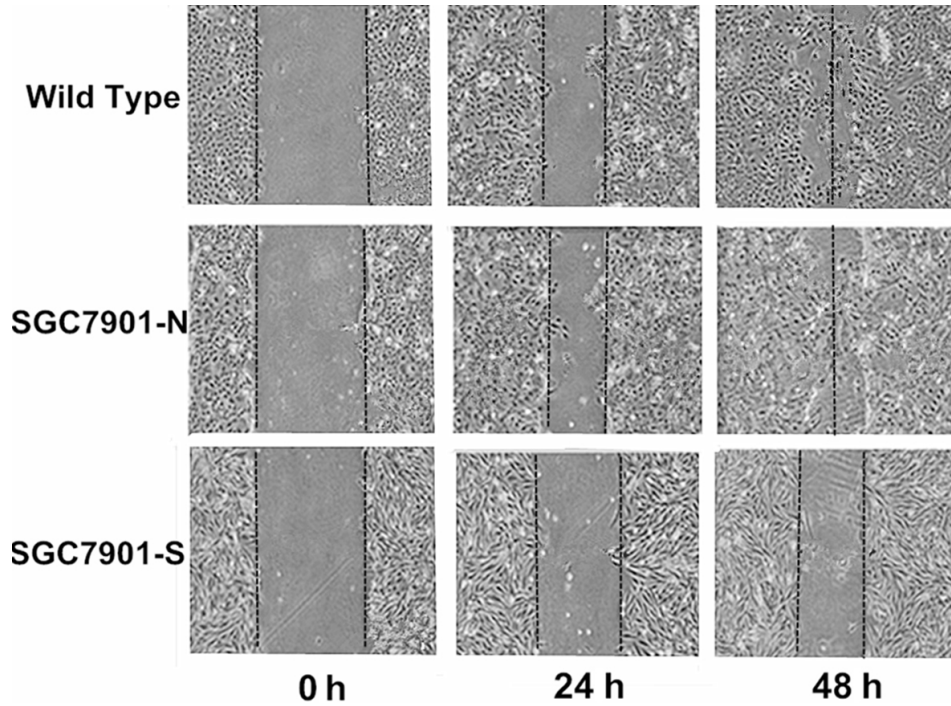


Figure 6. Scratch test.

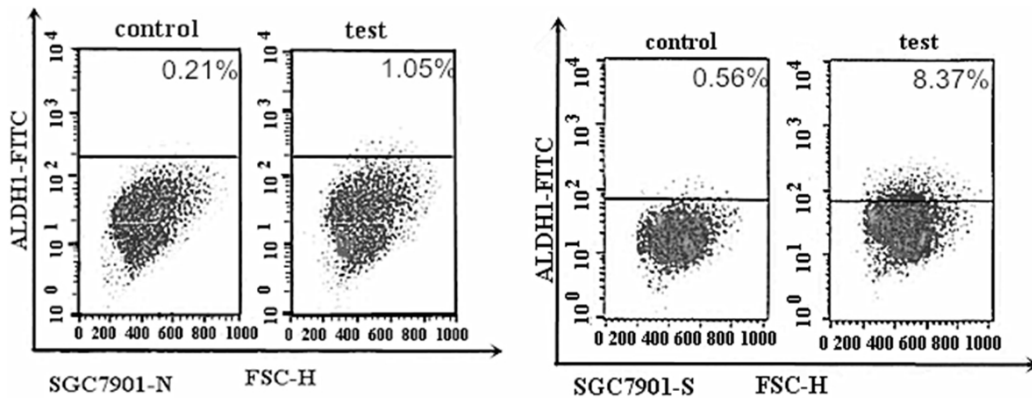


Figure 7. Flow cytometric analysis of positive expression of aldehyde dehydrogenase-1 (ALDH-1) in SGC7901 cells.

DISCUSSION

Despite the popularization of the concept of comprehensive treatment, the 5-year survival rate of patients with stomach cancer has increased substantially in recent years. However, tumor recurrence and distant metastasis are still the major causes (90%) of treatment

failure and death (Welte et al., 2015). The causes for stomach cancer invasion and metastasis remain to be determined, and CSCs evidently affect tumor metastasis and reoccurrence. A recent study has reported that tumor cells could turn into CSCs through EMT. Therefore, EMT and CSC-oriented treatment may increase the survival of cancer patients by decreasing tumor recurrence and metastasis.

EMT is regulated by various factors including Snail, a zinc-finger DNA binding protein. The 5882-bp long *Snail* gene is located at 20q12.3 in humans and contains 3 expressed regions. Snail is ubiquitously expressed in the placenta, heart, lungs, brain, liver, and bones. The zinc finger transcription factor Snail, first reported in *Drosophila melanogaster*, belongs to the Snail superfamily of transcription repressors. E-cadherin, a single transmembrane glycoprotein, is an important adhesion molecule present in the homotype epithelial cell, which plays a crucial role in maintaining the epithelial phenotype. Snail is mainly responsible for identifying and binding E-box sequences at the promoter site of E-cadherin and inhibiting the expression of E-cadherin, giving rise to EMT (Pez et al., 2011). It has been widely demonstrated that Snail increases tumor invasion and distant metastasis by facilitating EMT, an essential cause for poor prognosis (Moirangthem et al., 2016). However, the relationship between Snail and CSCs has not yet been revealed.

In this study, pEGFP-C1(-) and pEGFP-C1-Snail eukaryon plasmids (high-expression Snail plasmid) were transfected into SGC7901 cells, and stably expressed SGC7901-S and SGC7901-N cells were screened through their resistance to the G418 medium. We observed changes in the cell morphology from epithelial cell to fibroblast (from a tight, paving stone structure to a loose, elongated spindle structure with weaker adhesion capacity), which was consistent with the characteristics of mesenchymal cells. Functionally, fewer epithelial markers such as E-cadherin and β -catenin were expressed at the gene and protein levels, whereas more numbers of mesenchymal markers such as Snail and vimentin were expressed, as verified by the immunofluorescent staining. To sum up, overexpression of Snail induced EMT in SGC7901 cells. Based on the ectopic expression of Snail, Cano et al. (2000) confirmed that overexpression of Snail resulted in major morphological changes (fibroblast), loss of E-cadherin expression, increased expression of mesenchymal markers, and intensified malignancy, and invasive force, of tumor cells (Song et al., 2009; Ganesan et al., 2016). This is in accordance with the results obtained herein. Furthermore, the cell adhesion experiment confirmed that overexpression of Snail loosened the cell structure, weakened the cell adhesion, and drove SGC7901 cells further away, whereas the invasion assay indicated that Snail induced EMT and enhanced cell invasion in the SGC1709 cells.

Previous studies have reported that Snail-induced EMT leads to increased cell invasion, metastasis, and migration, thereby allowing tumor cells to separate from the original tissue. This results in local or distant metastasis of tumor cells. However, the scratch test showed no distinct change in the migration of SGC7901 cells after Snail overexpression, which may be attributed to the weakened proliferation of SGC7901 cells and their difficulty in migrating toward the scratches after overexpression. Accordingly, wild-type and SGC7901-N cells covered nearly the whole scratch after 48 h, whereas SGC7901-S cells failed to heal the wound and resembled some characteristics of CSCs. This was further confirmed by the FCM test. The rates of positive ALDH-1 expression in SGC7901-S and SGC7901-N cells were 8.37 and 1.05%, respectively, suggesting that the SGC7901-S group contained more ALDH-positive cells (CSCs).

ALDH-1, a catalytic cytoplasmic enzyme, catalyzes the metabolism of retinol into retinoic acid. It is vital to the regulation of cell proliferation and stem cell differentiation (Luo et al., 2012). ALDH-1 has been used to separate gastric CSCs (Nishikawa et al., 2013), in addition to its wide application in the separation of various CSCs (Hostettler et al., 2010; Prall et al., 2010; Alkatout et al., 2013). ALDH1⁺ diffuse gastric cancer cells have also shown stronger clonality and tumor formation ability, compared to ALDH1⁻ diffuse gastric cancer cells. Nishikawa et al. (2013) also reported that ALDH1⁺ cells could be resistant to chemotherapy. Similarly, ALDH-1 functions as an effective marker in gastric CSC sorting. Furthermore, the separation of cells with high ALDH activity in FCM is superior to that seen in conventional methods because of its simple operation, high repeatability, and mild toxic side effects on the marker substrate. In this study, ALDH1⁺ and ALDH1⁻ cells were separated successfully from among normal SGC7901 cells using a flow cytometer, further confirming that Snail-overexpressing gastric carcinoma cells developed certain characteristics of CSCs.

In summary, overexpression of Snail induced EMT in gastric carcinoma cells and led to the development of CSC characteristics, thereby providing a new target for stomach cancer treatment.

Conflicts of interest

The authors declare no conflict of interest.

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