RESEARCH COMMUNICATION

# Inhibition of Invasion and Metastasis of Gastric Cancer Cells Through Snail Targeting Artificial MicroRNA Interference

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## Abstract

**The zinc-finger factor Snai1 plays an important role in the down-regulation of E-cadherin expression and in the induction of epithelial-mesenchymal transition (EMT) during cancer progression. In gastric cancer tissues, we noted that Snail is abnormally high expressed and is remarkably associated with the lymph node metastasis. Using a plasmid containing newly synthesized artificial microRNA (amiRNA), we transfected gastric cancer cells to block Snail expression. Both Snail protein and mRNA levels were significantly decreased in stably transfected cells, while protein and mRNA expression of E-cadherin was up-regulated. In addition, migration and invasion potential were significantly decreased after knockdown of Snail.**

**Keywords:** Snail - E-cadherin - artificial microRNA - epithelial-mesenchymal transition - gastric cancer

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## Introduction

Gastric cancer is the second deadliest malignant neoplasm, with a 5-year survival rate of approximately 20% (Jemal et al., 2004). Invasion and metastasis of the cancer were the primary factors affecting the survival rate of patients (Hippo et al., 2001). Yet, the biochemical mechanisms that regulate invasion and metastasis remain poorly understood. Local invasion is the most important event prior to tumor metastasis. In the process of invasion, tumor cells undergo the mutation from epithelial to mesenchymal phenotype, which is termed as epithelial-mesenchymal transition (EMT), thus increasing cell mobility and promoting tumor cell invasion (Larue et al., 2005; Moreno-Bueno et al., 2008; Olmeda et al., 2008).

Since the discovery of EMT in tumors, much progress has been made towards understanding the mechanisms by which it occurs and towards the identification of factors involved in this process. E-cadherin plays an important role in cell to cell adhesion (Nurismah et al., 2008; Stepniak et al., 2009), and the decrease of E-cadherin expression is considered as the hallmark of EMT (Thiery et al., 2002). In the past decades, the molecular mechanisms of the down-regulation of E-cadherin expression during tumor progression have been well delineated (Christofori et al., 1999; Batlle et al., 2000), involving both genetic and epigenetic alternations, such as hypermethylation of E-cadherin promoter, chromosome remodeling, gene mutation and transcription repression. Among the alternations mentioned above, transcription repression has recently been proved to be the predominant mechanism in different human cancer cell lines. The zinc-finger factor Snai1, a key member of the Snail superfamily of transcriptional repressor, plays an important role in the down-regulation of E-cadherin expression and in the induction of EMT during embryogenesis and cancer progression (Batlle et al., 2000). Till now, numerous studies on the function of Snai1 in regulating E-cadherin expression have been published. Nevertheless, most of these studies have focused on its function in colon cancer (Pena et al., 2009), breast cancer (Van et al., 2011), and ovarian cancer (Blechschmidt et al., 2008). As for gastric cancer, little information regarding the role and mechanism of Snail in the process of metastasis of gastric cancer has been reported.

RNA interference has become an efficient approach to study the gene function in past years. Small interfering RNAs (siRNAs) and microRNAs (miRNAs) are two main types of small RNAs applied in RNA interference (Ossowski et al., 2008). The siRNA is released from a double-stranded RNA by RNaseIII-like enzymes, whereas the miRNA is released from a definite miRNA precursor by Dicer, in forms of 21-nucleotide RNA. By contrast with siRNA, miRNA could specifically bind target gene in both transcriptional and post-transcriptional regulation without off-target effects (Van et al., 2008). Artifical miRNAs (amiRNA), which was first used in human cell lines (Zeng et al., 2002) and later in Arabidopsis (Chung et al., 2006), was developed on the basis of endogenous miRNA. The efficiency of amiRNA in blocking the gene expression has been well supported by previous studies. In the current study, we observed the expression of the transcription factor Snail and E-cadherin in the gastric cancer tissues, as well as in the surrounding normal

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**Table 1. Sequences of Designed Oligonucleotides**

| Oligo | the sequence of DNA Single-Stranded oligomerization (5' to 3') |
|---|---|
| MR135-1-F | TGCTGTGGATTAGAGTCCTGCAGCTCGTTTTGGCCACTGACTGACGAGCTGCAACTCTAATCCA |
| MR135-1-R | CCTGTGGATTAGAGTTGCAGCTCGTCAGTCAGTGGCCAAAACGAGCTGCAGGACTCTAATCCAC |
| MR135-2-F | TGCTGAGGACAGAGTCCCAGATGAGCGTTTTGGCCACTGACTGACGCTCATCTGACTCTGTCCT |
| MR135-2-R | CCTGAGGACAGAGTCAGATGAGCGTCAGTCAGTGGCCAAAACGCTCATCTGGGACTCTGTCCTC |
| MR135-3-F | TGCTGAGAAGGTCCGAGCACACGCCTGTTTTGGCCACTGACTGACAGGCGTGTTCGGACCTTCT |
| MR135-3-R | CCTGAGAAGGTCCGAACACGCCTGTCAGTCAGTGGCCAAAACAGGCGTGTGCTCGGACCTTCTC |
| MR135-4-F | TGCTGTGAGCAGCCGGACTCTTGGTGGTTTTGGCCACTGACTGACCACCAAGACCGGCTGCTCA |
| MR135-4-R | CCTGTGAGCAGCCGGTCTTGGTGGTCAGTCAGTGGCCAAAACCACCAAGAGTCCGGCTGCTCAC |
| Negative-F | tgctgAAATGTACTGCGCGTGGAGACGTTTTGGCCACTGACTGACGTCTCCACGCAGTACATTT |
| Negative-R | cctgAAATGTACTGCGTGGAGACGTCAGTCAGTGGCCAAAACGTCTCCACGCGCAGTACATTTc |

gastric mucosa. Using the newly developed amiRNA, we generated a stably transfected cell line SGC-7901-amiRNA to investigate the relationship between Snail and E-cadherin in vitro. In addition, whether knockdown of Snail could efficiently suppress the invasion and metastasis of gastric cancer cells was also investigated.

## Materials and Methods

*Patients and samples*

Under the approval of the local ethics committee, a total of 212 patients from our center, who had undergone surgery for newly diagnosed gastric cancer between 2008 and 2010, were retrospectively reviewed. The informed consent was obtained from each patient. Samples of the primary gastric cancer tissues and the surrounding normal gastric mucosa were obtained in the surgery and then paraffin-embedded. According to Lauren's classification, all the samples were histologically verified by two experienced pathologists. The inclusion criteria were as follows: being primary tumor; having not received any therapy prior to surgery; being of diffuse-type gastric cancer. 74 patients met the criteria and were included in the study. There were 45 males and 29 females. The age ranged from 30 to 68 years old, averaged 58.2±5.7. Paraffin-embedded samples were immunohistochemically analyzed to detect the expression of Snail and E-cadherin. The expression of Snail and E-cadherin were compared between the gastric cancer tissues and normal gastric mucosa. The data of lymph node metastasis (LNM) was recorded for each patient.

*Immunohistochemistry*

All paraffin-embedded tissue sections (4 μm) were baked at 60°C for 6 hours. Slides were deparaffinized in xylene, rehydrated in graded alcohol, and washed in water. Endogenous peroxidase activity was blocked by incubation with 0.3% $H_2O_2$. Slides were placed in an autoclave cooker filled with 1 mmol/L EDTA (pH 8.0), and antigen retrieval was accomplished by steam heating for 10 minutes. After being treated with 10% normal goat serum for 25 minutes to block nonspecific protein binding, monoclonal antibody against E-cadherin (1:1000, Abcam, USA) and polyclonal antibody against Snai1 (1:1000, Abcam, USA) were applied to the slides at 4°C in a humid chamber overnight. After reaction with a mouse biotinylated secondary antibody, antigen-antibody reaction was visualized with diaminobenzidine serving as the chromogen. All slides were counterstained with hematoxylin. Breast cancer were used as positive control for E-cadherin and Snail, respectively. Negative controls were carried out by omission of the primary antibody. Two observers blinded to the clinical or molecular information of our study scored each slide independently.

*Immunohistochemical evaluation*

Expression of Snail1 and E-cadherin was assessed by a semi-quantitative scoring system (Blechschmidt et al., 2008). The scoring of Snail expression was listed as below: a score of 0 indicates no immunoreactivity or immunoreactivity in less than 1% of tumor cells; a score of 1+ indicates immunoreactivity in 1% of tumor cells; a score of 2+ indicates immunoreactivity in 2-5% of tumor cells; a score of 3+ indicates immunoreactivity in more than 5% of tumor cells. Snail staining was graded as positive only when nuclear staining was detectable. All cases were divided into two groups, with negative group representing cases of score 0 and with positive group representing cases of score 1+, 2+, and 3+.

The scoring of E-cadherin expression was listed as follows: a score of 0 indicates no immunoreactivity or immunoreactivity in less than 10% of tumor cells; a score of 1+ indicates low-intensity immunoreactivity in more than or equal to 10% of tumor cells; a score of 2+ indicates medium-intensity immunoreactivity in more than or equal to 10% of tumor cells; a score of 3+ indicates high-intensity immunoreactivity in more than or equal to 10% of tumor cells. All cases were divided into two groups, representing preserved E-cadherin expression (scoring 3+) or reduced E-cadherin expression (scoring 0, 1+, and 2+) respectively.

*Design of amiRNA and plasmid construction*

The pcDN6.2-GW/EmGFP-miR-based miRNA expressive plasmid (Invitrogen, Carlsbad, CA, USA), driving the expression of amiRNA with polymerase II and containing a spectinomycin resistant gene, was used in our study. The synthesized pre-miRNA sequence structure was based on the murine miR-155. According to the sequences of conserved region in Snail genome, we designed four target sequences against the S region of the Snail genome using Invitrogen RNAi design algorithm at https://rnaidesigner.invitrogen.com/rnaiexpress/. The BLAST algorithm was used to ensure the specifically targeting ability of the designed sequences. The oligonucleotides, as shown in Table 1, were annealed and ligated into

**Table 2. The Sequences of the Real-time PCR Primer Pair and the Fragment Size**

| Name | Sequence | Size |
|---|---|---|
| Snail1 | 5'-TATGCTGCCTTCCCAGGCTTG-3' | 143 bp |
| | 3'-ATGTGCATCTTGAGGGCACCC-5' | |
| E-cadherin | 5'-TCGACACCCGATTCAAAGTGG-3' | 194 bp |
| | 3'-TTCCAGAAACGGAGGCCTGAT-5' | |
| GAPDH | 5'-GGCGTTTTGCAATGCAGATGTAG-3' | 189 bp |
| | 3'-CACAGGAGCCGTCACTTCTCTTG-5' | |

pcDNA6.2-GW/EmGFP-miR vector. As a negative control, the pcDNA6.2-GW/EmGFP-miR-neg contained an insert that was predicted not to target any known human gene. Cells in the control group were mock-transfected with Lipofectamine 2000 alone.

*Cell culture and transfection*

Human gastric cancer cell line SGC-7901 was cultured in RPMI1640 medium (GIBCO/BRL) containing 10% fetal bovine serum (Hangzhou Sijiqing Biological Engineering Materials, China) and antibiotics (100 u/ml penicillin and 100 $\mu$g/ml streptomycin). Cells were amplified at 37 °C in a humidified incubator with 5% CO2 and 95% air (Thermo Direct Heat CO2, USA). Transfections were performed using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. Briefly, cells were trypsinized and plated in 6-well plates at a density of 1.0 × 105 cells/mL (1 mL/well) for 24 h before transfection. 10 µL of Lipofectamine was diluted by Opti-MEMI(Gibco, USA) for a final volume of 250 µL and incubated at room temperature for 5 min. 4.0 µg of amiRNA expression vector was also diluted by Opti-MEMI to a final volume of 250 µL and incubated at room temperature for 5 min. Then the diluted lipofectamine was mixed with the diluted amiRNA expression vector and incubated for another 20 min. The normal SGC-7901 cells and the cells transfected with negative control plasmid were used as the negative controls. After being cultured at 37 °C for 6h, the medium was completely removed and the cells were replenished with fresh medium. With the silencing effect evaluated by western blot, stably transfected cell clones were obtained by a long-term culture in a selected medium containing 5 mg/L Blasticidin.

*Total RNA extraction and Real-time PCR*

Total RNA was extracted and purified from $1{\times}10^6$ cells of SGC-7901, SGC-7901-Mock, and SGC-7901-miRNA with Trizol reagent (Invitrogen Corp, USA). cDNA synthesis was performed according to the manufacturer' s instructions (Invitrogen Corp). Amplification of 10% of these cDNAs by Real-time PCR was performed in a three-step thermal cycling incubations (30 s at 95 °C; 5 s at 95 °C; 34s at 60 °C) for 40 cycles, then in a final extension (15s at 95 °C, 1min at 60 °C, 15s at 95 °C) according to the manufacturer's recommendations (TaKaRa Corp). The sequences of the Real-time PCR primer pair and the fragment size are shown in Table 2.

*Western-blot analysis*

The whole cell lysates from SGC-7901, SGC-7901-Mock and SGC-7901-MiRNA were prepared in RIPA lysis buffer. Lysates from SGC-7901 and SGC-7901-Mock cells served as control. Lysates were then subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis on 10% or 7.5% acrylamide gels. The membranes were firstly incubated with primary antibodies at 4°C overnight, and then incubated with peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulins in Tris-buffered saline–Tween which contains 5% non-fat dry milk. At last, the membranes were processed with the enhanced chemiluminescence reagents according to the manufacturer's instructions. The expression of GAPDH was used as an internal control.

Cell migration and invasion assays

Cell migration and invasion assays were performed and evaluated as expounded in a standard protocol (Romanelli et al., 2006). We used Boyden chambers with 0.8 mm porosity polyvinylpyrrolidone-free polycarbonate filters that were coated with nothing for cell migration assay, and for invasion assay when coated with 50 lg/ml of Matrigel solution. The migration and invasion potencies of the cell clones were quantified by counting crystal violet-stained cells that either migrated to the lower surface of polycarbonate filters or invaded Matrigel, with a Zeiss microscope (Olympus, USA) equipped with brightfield optics. For each filter, the number of cells in five randomly chosen fields was counted, and the mean value was determined.

*Statistical analysis*

Statistical comparisons were performed with the software package SPSS 16.0. All the data are presented as mean ± standard deviation (SD). Mean differences were compared using traditional analysis of variance, and student t test was used for two-group comparisons. A p value of less than 0.05 was considered statistically significant.

## Results

*Immunohistochemical detection of Snail and E-cadherin in gastric cancer tissues and surrounding normal gastric mucosa*

Using immunohistochemistry analysis, we found that Snail was over-expressed in primary human gastric cancer. Among the gastric cancer tissues of 74 patients, 42(56.8%) samples showed positive Snail expression. The reduced E-cadherin expression was detected in 36(48.7%) samples. In the normal gastric mucosa, we failed to detect Snail expression, while all these samples showed E-cadherin expression. Among samples of the 48 patients with LNM, 33(68.7%) showed a positive Snail expression. Statistic analysis showed that the LNM incidence in Snail positive samples was remarkably higher than in Snai1 negative ones (78.5% vs. 46.8%, p=0.019).Representative results of Snail and E-cadherin staining are shown in Figure 1.

*Detection of protein and mRNA expression of both Snai1 and E-cadherin after transfection*

Once the recombinant plasmids were transfected into SGC-7901 cells, the expression of Emerald green

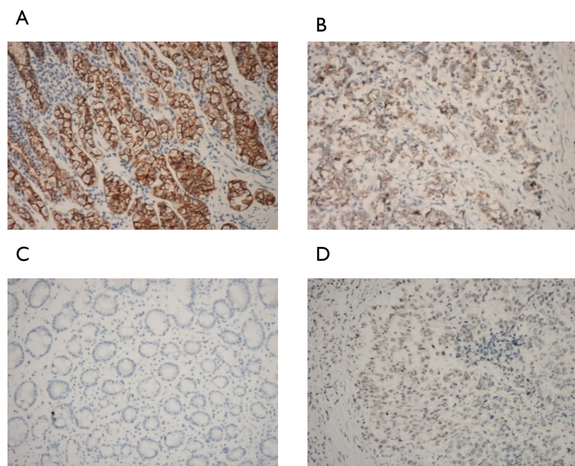



**Figure 1. Representative Photographs of Tissue of Human Gastric Cancer and Corresponding Normal Gastric Mucosa were Stained by Immunohistochemically for E-cadherin** (A, B) and Snail (C, D) expression. A, C: normal gastric mucosa; B, D: gastric cancer tissue

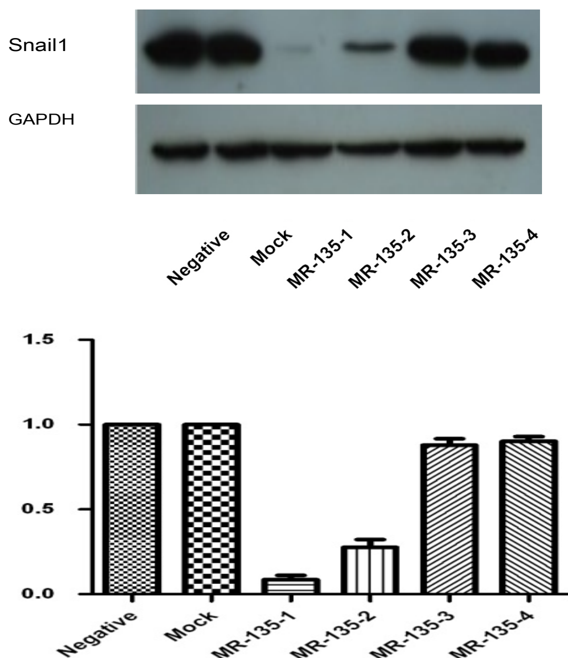
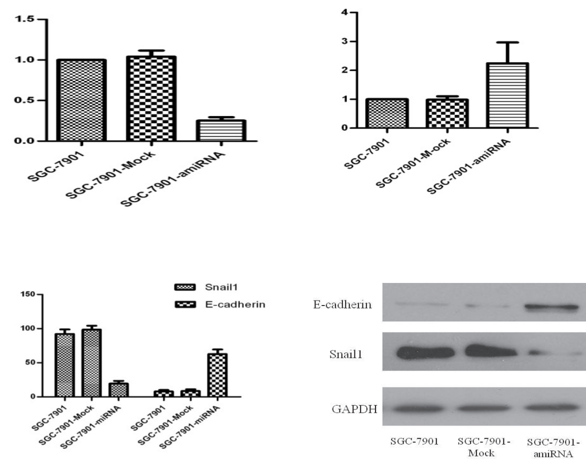



**Figure 2. Effect of AmiRNA-Snail with Transient Transfection on Snail Protein Levels.** Both MR-135-1 and MR-135-2 had significant inhibitory effect on Snail protein (p<0.01)

fluoresecent protein (EmGFP) could be observed directly under fluorescence microscope. After cultured for 48h, we found that the transfection efficiency of these vectors into SGC-7901 cells were over 75%, and the expression of Snai1 proteins could be detected by western blot. There was no significant difference between parental cells and cells transfected with mock vector. Compared with parental cells, both MR-135-1 and MR-135-2 resulted in a significantly reduced level of Snai1 protein in SGC-7901-amiRNA (p<0.01) (Figure 2).

MR-135-1 amiRNA was more efficient than MR-135-2 amiRNA, with 91.5% inhibitory rate in Snai1 protein. Therefore, MR-135-1 amiRNA transfected cells and mock vector transfected cells were selected by Blasticidin,



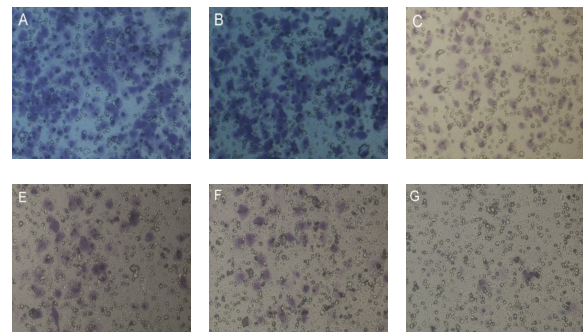




**Figure 3. mRNA Expression of Human Snail** (A) and E-cadherin (B) in parental SGC-7901 and SGC-7901-Mock, SGC-7901-amiRNA stable clones; (C) Protein expression of snail and E-cadherin in the same cell clones as those in RT-PCR analysis. GAPDH served as the loading control

**Figure 4. Snail1 Stably Silenced Cells Showed Lower Migration and Invasion Ability.** A-C: migration analysis E-G: invasion analysis. A, E: SGC-7901 B, F: SGC-7901-Mock C, F: SGC-7901-amiRNA

and stably transfected cells (SGC-7901-amiRNA and SGC-7901-Mock) were obtained. The effectiveness of Snail interference in the stable clones was determined by RT-PCR. As shown in Fig.3A, low mRNA expression (< 10% of the corresponding control) of Snail was observed in SGC-7901-amiRNA clones, while SGC-7901-Mock cells showed normal Snail mRNA expression similar to the parental SGC-7901 cells. Analysis of E-cadherin mRNA expression levels (Figure 3B) indicated up-regulation of E-cadherin transcription in cells with silenced Snail expression. Up-regulation of E-cadherin protein expression in the SGC-7901-amiRNA clones was further confirmed by Western blot (Figure 3C).

*The effect of Snail knockdown on the migration and invasion potencies of gastric cancer cells*

The results of Transwell experiment showed that the number of cells in down-pore of uncoated micro-membrane was 20±4.2 in the transfected group, significantly less in comparison with 105±7.6 in parent cells group and 110±9.2 in mocked cells group (P <0.05). For micro-membrane coated with Matrigel solution, we detected a remarkable reduction of the number of cell in all groups. The cell number in the transfected group was significantly less than in mocked cells group and in parent cells group (5.0±1.2 vs. 15.2±2.1 & 16.3±1.7, p<0.05).

## Discussion

Cancer cell metastasis is mediated by increased mobility and invasiveness via EMT (Jechlinger et al., 2003). Snail, a zinc-finger transcription factor, was reported to trigger EMT by directly repressing E-cadherin expression (Nakayama et al., 1998). The current study has shown that Snail is abnormally high expressed in the gastric cancer tissues, and is remarkably associated with the LNM. Through in vitro study, we found that there exists an inverse correlation between Snail and E-cadherin mRNA and protein production in gastric cancer cell lines. Inhibition of the Snail expression could result in the high expression of E-cadherin, which had a significant influence on the metastatic property of the cancer cells.

The process of EMT is always accompanied by the loss of cell-cell adhesion mediated by E-cadherin, which plays a critical role in the maintenance of cell polarity and epithelial integrity (Perl et al., 1998). E-cadherin transcription in vitro and in vivo could be repressed by the zinc-finger transcription factor Snail, which was reported to bind to the sequence in the E-cadherin promoter (Thiery et al., 2002). Snail-expressing cells became invasive, supporting its role in tumor progression (Barbera et al., 2004). To date, over expression of Snail has been extensively investigated in different human cancer tissues and cell lines (Blechschmidt et al., 2008; Pena et al., 2009; Van et al., 2011). Elmoneim and Zaghloul (2011) used Immunohistochemistry to examine Snail protein expression in 132 invasive breast carcinomas. In their study, Snail was detected in approximately 40% of the carcinoma tissue, while it was negative in normal glandular structures around the tumor. In present study, we have a similar finding with previous studies, that Snail is significantly high expressed in cancer tissues than in normal tissue. The protein expression of Snail was detected in 56.8 % of the whole gastric cancer tissues. By contrast, absent Snail expression was found in the normal surrounding gastric mucosa. In addition, a correlation between Snail expression and lymph node status was detected in our study. We found that Snail remarkably increased in node-positive cancer tissue, when compared with its expression in node-negative cancer. This result is in agreement with previous results (Blanco et al., 2002; Elmoneim et al., 2011), which showed that Snail expression correlates with the presence of lymph node metastases. Moreover, our results also revealed an inverse correlation between protein expression of Snail and E-cadherin. This finding is in line with the report of Rosivatz et al. (2002), who firstly provided the evidence that an increase in Snail mRNA expression is associated with down-regulation of E-cadherin in gastric cancer. Interestingly, in some samples with absence or low-intensity of Snail expression, E-cadherin expression was found to be at a low level. Earlier studies (Rosivatz et al., 2002; Castro et al., 2007) have revealed that some other E-cadherin repressors, including Twist, Slug, and SIPI, could result in a decreased expression of E-cadherin. In gastric cancer cell, it's very likely that these repressors regulated the expression of E-cadherin together with Snail, as indicated by our results. Further study into the co-efficiency of these repressors is still warranted, so as to clearly clarify the regulation of E-cadherin and occurrence of EMT.

Through immunohistochemical assay of the gastric cancer tissues, we substantiated that Snail plays a critical role in EMT and the subsequent cancer metastasis. We hypothesized that Snail inhibition may serve as a promising target for therapeutic strategies to prevent cancer metastasis. Thus, a knowledge of the molecular mechanisms that control its expression or function is of great importance in understanding the process of cancer invasion. To validate this hypothesis, and to further investigate the role and mechanism of Snail in the process of metastasis of gastric cancer, we studied the gene function of Snail using RNA interference induced by amiRNA. amiRNA have been proved to be a promising tool applied in the cancer research (Liang et al., 2007; Wang et al., 2008), mainly attributed to its high efficiency and specificity in the knockdown of target gene. Recently, a new RNA polymerase II expression vector based upon murine miR-155 sequence came into use (Chung et al., 2006). In the current study, we constructed miR155-based Snail-targeting pre-miRNA pol II vectors. Using this newly synthesized miRNA, we obtained stably transfected gastric cancer cell lines. The results of RT-PCR and western blot showed that both Snail protein and mRNA levels were significantly decreased in pcDNA.Snail-miR group. Meanwhile, the protein and mRNA expression of E-cadherin was up-regulated. Besides, according to the results of the migration and invasion potency assay of the cancer cells, we found that knockdown of Snail was significantly associated with the cancer cell metastasis. Namely, effective and specific blockade of Snail could result in a remarkable change of the expression of E-cadherin, which in turn reversed the process of EMT and decreased the invasion potency of the cancer cell.

Some recent studies have made use of several siRNA duplex oligonucleotides against Snail to assess the effect of transient silencing of the factor (Espineda et al., 2004; Kajita et al., 2004). Olmeda et al. (2007) blocked Snail with shRNA and reported the resultant down-regulation of E-cadherin and reduction of the tumor growth. It is the first time, to our knowledge, that stable silencing of Snail expression in gastric cancer cells was achieved by amiRNA. Our study evidenced that amiRNA could efficiently reverse the expression ratio of Snail to E-cadherin and prevent cancer metastasis, and this may shed light on the clinical treatment of cancer.

Some limitations still exist in our study. First, there was only one gastric cancer cell line used in the current study, which might lead to the insufficiency in illustrating the role of Snail in human cancer progression and metastasis. Second, an in vivo study is quite essential for the substantiation of the efficiency of the currently used amiRNA in inhibiting the migration and invasion potencies of gastric cancer. Third, the 5-year survival rate of the patients could not be collected in the current study, as a result of relatively short follow-up period. In the future study, this clearly needs to be taken into consideration, so as to provide a better evaluation of the clinical significance of Snail.

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