

ETV1 induces epithelial to mesenchymal transition in human gastric cancer cells through the upregulation of Snail expression

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Received July 25, 2013; Accepted September 17, 2013

DOI: 10.3892/or.2013.2776

Abstract. The ETS family of transcription factors is involved in several physiological and pathological processes including tumor progression. The ETS transcription factors are divided into subfamilies based on the sequence and location of the ETS domain. ETV1 (Ets variant gene 1; also known as ER81), is a member of the PEA3 subfamily, which has been found to promote metastatic progression in several types of human cancer. Previous findings demonstrated that ETV1 expression is upregulated in gastric adenocarcinomas; however, the underlying mechanisms of ETV1-induced metastatic progression in gastric cancer remain elusive. In the present study, we found that the overexpression of ETV1 in normal gastric epithelial cells resulted in epithelial to mesenchymal transition (EMT) and increased invasiveness. Conversely, knockdown of ETV1 resulted in decreased aggressiveness of the invasive gastric cancer cells. Mechanistically, ETV1 transcriptionally upregulates Snail expression. Of note, ETV1 expression is significantly correlated with Snail expression in human gastric tumor samples. In summary, we present data that ETV1 promotes Snail expression to induce EMT-like metastatic progression in gastric cancer.

Introduction

Gastric cancer is one of the most frequently occurring types of cancer in China (1). Metastatic disease is one of the major causes of mortality in cancer patients, and exploring the mechanisms that are the acquisition of a metastatic phenotype may offer new therapeutic strategies for metastatic gastric cancer patients.

The ETS family of transcription factors is involved in several physiological and pathological processes including

tumor progression (2). The ETS transcription factors are divided into subfamilies based on the sequence and location of the ETS domain. ETV1 (Ets variant gene 1; also known as ER81), is a member of the PEA3 subfamily, which has been found to promote metastatic progression in several types of human cancer. In prostate cancer, ETV1 performs its oncogenic effects through upregulating the matrix metalloproteinase-7 (MMP-7) gene expression (3); ETV1 has also been shown to cooperate with the androgen receptor (AR) to bind to the prostate-specific antigen enhancer and enhance gene transcription (4,5). In gastrointestinal stromal tumor (GIST), ETV1 was shown to be highly expressed and to be activated by KIT mutations (6), but this was disputed by another study recently (7). Although previous studies demonstrated that ETV1 expression was upregulated in gastric adenocarcinomas (8,9), the underlying mechanisms of ETV1-induced metastatic progression in gastric cancer remain elusive.

Epithelial-mesenchymal transition (EMT) is a process characterized by loss of cell-cell adhesion, repression of E-cadherin expression and gain of cell motility (10). In cancer cells, the EMT process may promote their metastatic potential. Transcription factors, including Snail, Slug and TWIST, have been demonstrated to be master regulators of EMT (11). These proteins are transcriptional repressors of E-cadherin and their expression induces EMT, thereby increasing motility and invasiveness of cancer cells.

The role of Snail in EMT and metastatic progression of cancer cells has been extensively studied (12). In the present study, we present data showing that ETV1 promotes Snail expression to induce EMT-like metastatic progression in breast cancer.

Materials and methods

Cell culture and transfection. Human normal gastric epithelial cell line GES-1, gastric cancer cell lines MKN-45, MGC-803 and SGC-7901, HEK 293T cells were maintained and cultured in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen). All cell lines were incubated at 37°C in humidified air with 5% CO₂.

Cells were transfected with Flag-tagged ETV1 using Lipofectamine™ 2000 reagent (Invitrogen), according to

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Key words: epithelial to mesenchymal transition, ETV1, Snail, gastric cancer

the manufacturer's protocol. siRNA oligonucleotides against human ETV1 (ON-TARGETplus SMARTpool) and non-targeting siRNAs control oligonucleotides were obtained from Dharmacon using DharmaFECT1 siRNA transfection reagent (Dharmacon).

Western blot analysis. Whole cell extracts were prepared using Laemmli Buffer. Samples were run on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred to a nitrocellulose membrane. Membranes were blocked in 5% milk solution [Tris buffered saline (TBS)-0.1% Tween] for 1 h at room temperature and incubated with indicated primary antibody in 5% milk solution overnight at 4°C. The membranes were washed three times for 10 min in TBS-0.1% Tween at room temperature and incubated for 1 h with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibody. Proteins were detected by the enhanced chemiluminescence system (Amersham Pharmacia Biotech) as described by the manufacturer's instructions. The primary antibodies used for western blot analysis were: anti-ETV1 (ab81086; Abcam), anti-Snail (sc-10432), anti-GAPDH (sc-25778; Santa Cruz Biotechnology), anti-Vimentin (550513), anti-N-cadherin (610921), anti-E-cadherin (610182), anti- β -catenin (610153; BD Transduction Laboratories).

mRNA isolation and quantitative PCR (qPCR). mRNA was isolated using TRIzol (Invitrogen) and cDNA was prepared using transcript first-strand cDNA synthesis kit (Roche) according to the manufacturer's protocol. qRT-PCR was carried out using SYBR-Green (Roche) on a MasterCycler RealPlex4 instrument. Gene expression was normalized to *Gapdh*. The upstream and downstream primers used for ETV1 gene were: 5'-TACCCATGGACCACAGATT-3' and 5'-CACTGGGTCGTGGTACTCCT-3'.

Transwell invasion assay. Transwell invasion experiments were performed with 24-well Matrigel-coated chambers (8 μ m pore size) from BD Biosciences. Briefly, cells were allowed to grow to subconfluency (~80%) and were serum-starved for 24 h. After detachment with trypsin, cells were washed with phosphate-buffered saline, resuspended in serum-free medium and 5×10^4 cells were added to the upper chamber. Complete medium was added to the bottom wells of the chambers. After 12 h, the cells that had not migrated were removed from the upper face of the filters using cotton swabs, and the cells that had migrated were fixed and stained by crystal violet solution.

Luciferase assay. HEK 293T cells were co-transfected with the indicated plasmids. TK-*Renilla* expression plasmid was used as an internal control. Luciferase activity was measured after 24 h using a dual luciferase assay kit according to the manufacturer's protocol (Promega). Statistical analysis was performed using GraphPad Prism 4.0.

Chromatin immunoprecipitation (ChIP). The ChIP kit was obtained from Millipore and ChIP was performed according to the manufacturer's instructions. Immunoprecipitation was carried out using 2 mg of ETV1 antibody, 2 mg normal rabbit IgG (Sigma-Aldrich). PCR was carried out using the following

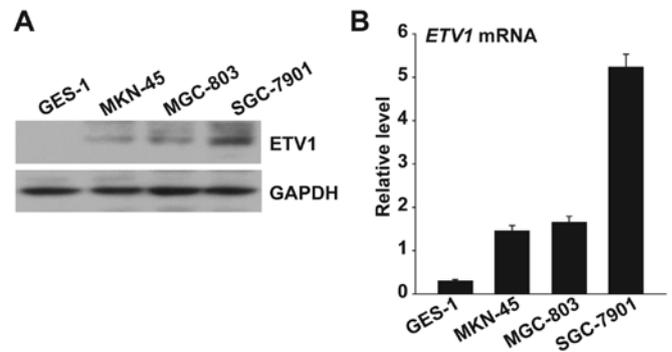


Figure 1. Expression of ETV1 in normal gastric epithelial and gastric cancer cell lines. (A) Immunoblot analysis of ETV1 levels in human normal gastric epithelial cell line GES-1 and the gastric cancer cell lines (MKN-45, MGC-803 and SGC-7901). GAPDH was detected as a loading control. (B) The transcript levels of ETV1, relative to *Gapdh*, determined by qRT-PCR; error bars indicate SEM; n=3 experiments.

primers: forward, 5'-CCAGTGATGTGCGTTTCCCT-3' and reverse, 5'-AAGCGAGGCTCTGCGAGGT-3'.

Immunohistochemistry. Expression of ETV1 and Snail was analyzed using human gastric cancer tissues from 20 patients in grade I and 20 patients in grade III. Use of the tissue samples was approved by the Harbin Medical University Institutional Review Board. Standard immunohistochemical procedures were carried out using an anti-ETV1 or anti-Snail antibody. The staining results were scored by two investigators blinded to the clinical data. As negative controls, the primary antibodies were omitted and replaced with a related strain of IgG used as a negative control.

Statistical analysis. The Student's t-test was used for two-group comparisons. Comparisons between three or more groups were analyzed by one-way ANOVA followed by the Duncan's test in SPSS 15.0 (SPSS Inc.). The probability of $P < 0.05$ was considered to indicate a statistically significant result.

Results

Expression of ETV1 in normal gastric epithelial and gastric cancer cell lines. First, we determined the ETV1 expression level using qPCR and western blot analysis in human normal gastric epithelial cell line GES-1 and the gastric cancer cell lines (MKN-45, MGC-803 and SGC-7901). The level of ETV1 expression in the three gastric cancer cell lines was significantly higher than in the normal gastric epithelial cell line GES-1 at both the protein and the mRNA level (Fig. 1). The highest levels of ETV1 expression were observed in the SGC-7901 gastric cancer cell line, which is a high metastatic potential cell.

Overexpression of ETV1 in normal gastric epithelial cells results in EMT and increased invasiveness. GES-1 cells overexpressing ETV1 expressed higher levels of mesenchymal markers, such as vimentin and N-cadherin, and lower levels of epithelial markers, such as E-cadherin and β -catenin, compared with their vector control (Fig. 2A). To investigate whether the ETV1-induced EMT-like phenotype could be translated into enhanced metastatic ability of the GES-1 cells,

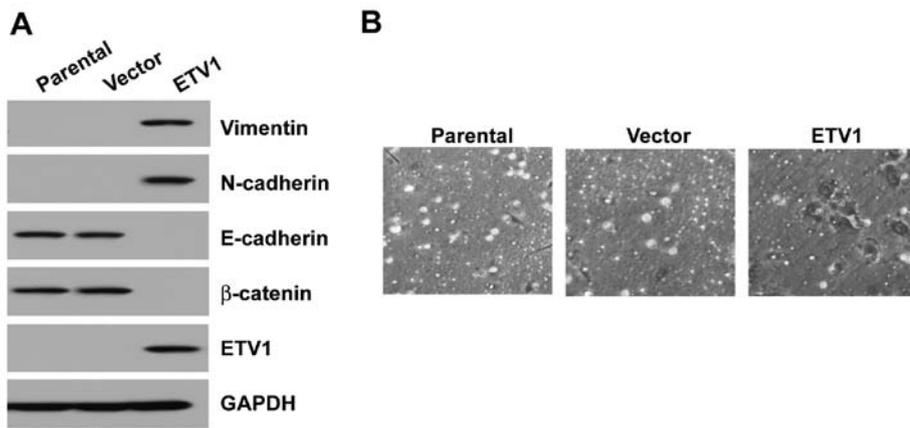


Figure 2. Overexpression of ETV1 in normal gastric epithelial cells results in EMT and increased invasiveness. (A) The expression of epithelial or mesenchymal markers was detected in the overexpressing of ETV1 GES-1 cells by western blot assay. (B) Cell invasion was measured using Transwell assays for parental, Control and ETV GES-1 cells.

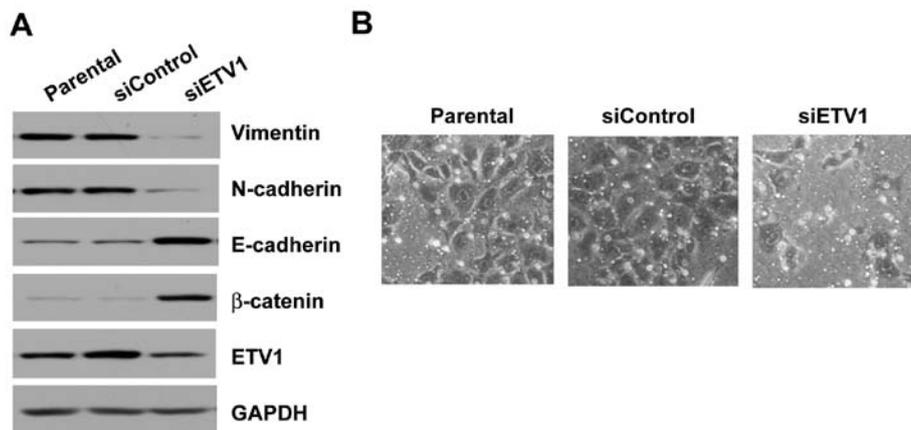


Figure 3. Knockdown of ETV1 results in decreased aggressiveness of the invasive gastric cancer cells. (A) The expression of epithelial or mesenchymal markers was detected in the knockdown of ETV1 SGC-7901 cells by western blot assay. (B) Cell invasion was measured using Transwell assays for parental, siControl and siETV SGC-7901 cells.

the invasion of ETV1 cells was tested. ETV1-overexpressing GES-1 cells displayed an increase in motility through the Matrigel in Transwell invasion assays, compared with the vector control cells (Fig. 2B).

Knockdown of ETV1 results in decreased aggressiveness of the invasive gastric cancer cells. ETV1 was knocked down by siRNA in the high metastatic potential cell line SGC-7901, which expresses high levels of ETV1. Knockdown of ETV1 in the cell line resulted in a significant decrease at the protein level. Protein expression of the mesenchymal marker vimentin and N-cadherin was also decreased. The level of E-cadherin and β -catenin, both epithelial markers, was induced (Fig. 3A). Furthermore, knockdown of ETV1 in SGC-7901 cells resulted in decreased ability of the cells to invade through the Matrigel in the invasion assay (Fig. 3B).

Overexpression of ETV1 promotes Snail expression but knockdown of ETV1 inhibits it. To elucidate the mechanism of ETV1 that leads to EMT and invasiveness, we hypothesized that ETV1 regulates the expression of Snail. Indeed, ectopic ETV1 expression greatly increased Snail at both the

mRNA and the protein levels. Conversely, knockdown of ETV1 reduced the Snail expression (Fig. 4A and B). Next, we assessed whether ETV1 promotes the activity of the *Snail* gene promoter. To test this possibility, the Snail luciferase reporter construct (*Snail*-Luc) transiently transfected into HEK 293T cells with and without ETV1 expression plasmids. Luciferase reporter activity increased in a dose-dependent manner in cells with ectopic ETV1 expression compared with empty vector (Fig. 4C). This result suggested that ectopic ETV1 expression promotes the promoter activity of *Snail*. To determine if ETV1 interacted with the endogenous *Snail* promoter, we performed ChIP assays. Primers flanking the predicted ETV1 binding site of the *Snail* promoter were used to amplify chromatin fragments enriched by ETV1 binding to this region. The PCR products were amplified from DNA fragments immunoprecipitated with an anti-ETV1 antibody but not from DNA fragments precipitated with an IgG control antibody. As shown as Fig. 4D, ETV1 bound to the *Snail* promoter.

Correlative expression of ETV1 with high levels of Snail in gastric adenocarcinoma specimens. To examine whether an increase in ETV1 expression was associated with an increase

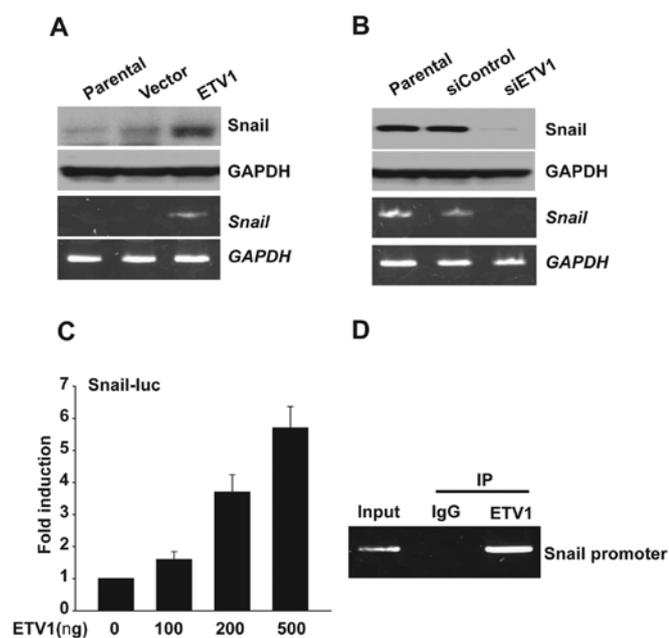


Figure 4. Overexpression of ETV1 promotes Snail expression but knockdown of ETV1 inhibits it. (A) Overexpression of ETV1 increased the expression of Snail at both the mRNA and the protein level in GES-1 cells. (B) Knockdown of ETV1 decreased the expression of Snail at both the mRNA and the protein level in GC-7901 cells. (C) HEK 293 cells were transfected with the Snail-luc reporter construct for 24 h. The cells were harvested and luciferase activity was measured. (D) Chromatin immunoprecipitation (ChIP) assay of Snail promoter DNA using PCR.

in Snail level in human gastric tumor samples, we performed immunohistochemistry to assess the expression of ETV1 and Snail at the protein levels from 40 gastric adenocarcinomas. We found that the protein levels of both molecules were significantly correlated (Fig. 5).

Discussion

ETV1 belongs to the PEA3 subfamily, which is associated with a variety of cancers including colon, breast, prostate and gastric cancer (6,13-15). In the presents study, we showed that ETV1 transcriptionally activates Snail, thereby promoting EMT and invasive progression *in vitro*. In human specimens, we further confirmed that the expression levels and nuclear localization of ETV1, as well as that of Snail, are potential prognostic markers for gastric cancer patients.

In the present study, we first examined the expression of ETV1 in normal gastric epithelial and gastric cancer cell lines at the mRNA level by real-time PCR and at the protein level by western blotting. EMT is a process that enables cancer cells to lose their cell-cell and cell-matrix contacts to gain migratory properties through transcriptional reprogramming (16,17). To demonstrate the role of ETV1 in EMT, we silenced the expression of ETV1 using siRNA in SGC-7901 cells with highly invasive potential and ETV1 high expression. Moreover, we also overexpressed ETV1 in normal gastric epithelial cell line GES-1 without invasion and ETV1 expression. We found that the variation of ETV1 expression significantly correlates with several putative EMT marker expressions. In addition, assessment of the invasive potential, following transfection

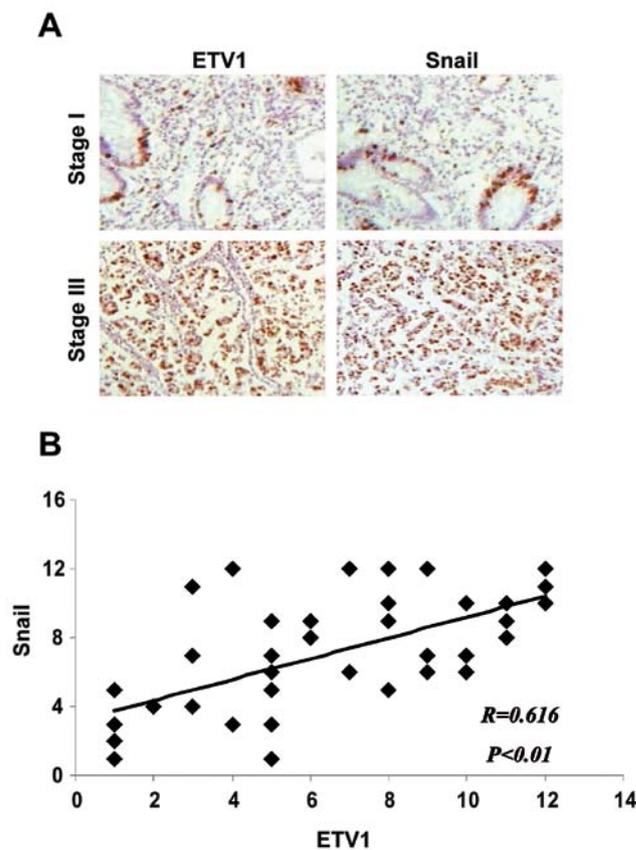


Figure 5. Correlative expression of ETV1 with high levels of Snail in gastric adenocarcinoma specimens. (A) The expression of ETV1 and Snail at the protein levels from 40 gastric adenocarcinomas by immunohistochemistry assay. (B) ETV1 expression is significantly correlated with Snail expression in human gastric tumor samples.

with ETV1-siRNA, indicated that the rate of cell invasion was markedly reduced compared to those in siControl and mock control, suggesting that ETV1 contributes to the invasive potential of gastric cancer cells.

Although ETV1 overexpression is thought to contribute to gastric carcinogenesis, it has remained unknown by which molecular mechanism the transcription factor achieves its deleterious effects. It is well-known that Snail is required for EMT-mediated metastatic development and its upregulation is associated with recurrence (18). The present study provides a mechanistic explanation in the case of ETV1, i.e., through inducing transcription as exemplified for the Snail gene.

Immunohistochemical expression of ETV1 was associated with an increase in the tumor grade and tumor stage suggesting that increasing levels of ETV1 in primary tumors are associated with a more differentiated phenotype and initial stages of carcinogenesis. ETV1 expression was significantly correlated with Snail expression, further supporting the hypothesis that ETV1 plays an important role in the induction of Snail expression.

In conclusion, the present study is the first to show that ETV1 may induce EMT to acquire invasion in gastric cancer cells. The function of ETV1 as an oncogene may be associated with several important molecules involved in the invasion of cancer cells. These results further suggest that ETV1 may serve as a potential target for the development of therapies for gastric cancer.

Acknowledgements

The present study was supported by the Foundation of Educational Commission of Heilongjiang Province, China (11541224) to Z.L.

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