Abstract

**Background:** Epithelial–mesenchymal transition (EMT) plays a significant role in tumor progression and invasion. Snail is a known regulator of EMT in various malignant tumors. This study investigated the role of Snail in gastric cancer.

**Methods:** We examined the effects of silenced or overexpressed Snail using lenti-viral constructs in gastric cancer cells. Immunohistochemical analysis of tissue microarrays from 314 patients with gastric adenocarcinoma (GC) was used to determine Snail’s clinicopathological and prognostic significance. Differential gene expression in 45 GC specimens with Snail overexpression was investigated using cDNA microarray analysis.

**Results:** Silencing of Snail by shRNA decreased invasion and migration in GC cell lines. Conversely, Snail overexpression increased invasion and migration of gastric cancer cells, in line with increased VEGF and MMP11. Snail overexpression (≥75% positive nuclear staining) was also significantly associated with tumor progression (P < 0.001), lymph node metastases (P = 0.002), lymphovascular invasion (P = 0.002), and perineural invasion (P = 0.002) in the 314 GC patients, and with shorter survival (P = 0.023). cDNA microarray analysis revealed 213 differentially expressed genes in GC tissues with Snail overexpression, including genes related to metastasis and invasion.

**Conclusion:** Snail significantly affects invasiveness/migratory ability of GCs, and may also be used as a predictive biomarker for prognosis or aggressiveness of GCs.

**Keywords:** Stomach, Adenocarcinoma, Snail, Lymph node metastasis, Survival
the roles of Snail in GC have included clinicopathological, prognostic, and functional in vitro analyses as well as gene expression results. We therefore evaluated Snail’s effect on invasiveness/migratory ability in gastric cancer cell lines, and also investigated the possibility of Snail being used as a predictive marker for evaluating poor prognosis or tumor aggressiveness in GC patients. We also evaluated the gene expression pattern in 45 GC tissues with Snail overexpression, using cDNA microarrays.

Methods

shRNA lentivirus-mediated silencing and overexpression of Snail in gastric cancer cells

Human gastric cancer cell lines SNU216 and SNU484 were obtained from Korean Cell Line Bank (KCLB) and were authenticated by DNA profiling. These cells cultured in RPMI1640 medium with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin (hyClone, Ogden, UT). All cells were maintained at 37°C in 5% CO₂. Lentiviral-based RNA knockdown and overexpression were used for silencing and overexpression of Snail. Lentiviruses expressing either non-target or Snail-targeted shRNAs were used for silencing; a PLKO lentiviral vector targeting Snail or an empty PLKO vector were used for overexpression of Snail in the SNU216 and SNU484 cells. Lentivirus stocks were produced using the Virapower™ lentiviral packaging mix using the 293FT cell line according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). SNU216 and SNU484 cells grown to 50% confluence were incubated for 24 h in a 1:1 dilution of virus/media with 5 μg/ml Polybrene. After a 24-h recovery period in complete media without virus, polyclonal stable cell lines were selected and maintained in media containing 5 μg/ml puromycin. Silencing or overexpression of Snail was determined by RT-PCR and western blotting.

Real time RT-PCR analysis of VEGF, MMP11, and Snail in gastric cancer cells

Total cellular RNA was extracted using the TRIzol method (Sigma-Aldrich, St Louis, MO, USA). For RT-PCR analysis, 2-μg aliquots of RNA were subjected to cDNA synthesis with 200 U of MMLV reverse transcriptase and 0.5 μg of oligo(dT)-15 primer (Promega, Madison, WI, USA). Quantitative real-time PCR was performed with the Rotor-Gene™ System (QIAGEN, Hilden, Germany) using AccuPower 2x Greenstar qPCR Master Mix (Bioneer, Daejeon, Korea). cDNA in 1 μl of the reaction mixture was amplified with 0.5 μl of GoTaq DNA polymerase (Promega) and 10 pmol each of the following sense and anti-sense primers: GAPDH 5'-TCTATGACAATCTGGATGC-3', 5'-TGATTGACCCACTTTGTC-3'; Snail 5'-CTTCCCTCTAATGATGGTAATGTTG-3', 5'-TGATTGAGGTAGTCACCACTCGT-3'; VEGF 5'-TTGCTGCTCTTACCCTACAC-3', 5'-GCACACAGGATTGGCTGAA-3'; MMP11 5'-CTTG

GCTGCTTTGTGTGCT-3', 5'-AGGTATGGAGCGATG

TGACG-3'. The thermal cycling profile was: denaturation for 30 s at 95°C, annealing for 30 s at 52°C (depending on the primers used), and extension for 30 s at 72°C. For semi-quantitative assessment of expression levels, 30 cycles were used for each PCR reaction. PCR products were size-fractionated on 1.0% ethidium bromide/agarose gels and quantified under UV transillumination. The threshold cycle (CT) is defined as the fractional cycle number at which the fluorescence passes a fixed threshold above baseline. Relative gene expression was quantified using the average CT value for each triplicate sample minus the average triplicate CT value for GAPDH. Differences between the control (empty vector) and experiment groups (infected with the lentivirus) were calculated using the formula 2⁻^{\Delta \Delta CT} [16] and expressed as a fold change in expression according to the comparative threshold cycle method (2⁻^{\Delta CT}) [16].

Western blotting

Cells were harvested and disrupted in lysis buffer (1% Triton X-100, 1mM EGTA, 1mM EDTA, 10mM Tris–HCl, pH 7.4 and protease inhibitors). Cell debris was removed by centrifugation at 10,000 × g for 10 min at 4°C. The resulting supernatants were resolved on a 12% SDS-PAGE under denatured reducing conditions and transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat dried milk at room temperature for 30 min and incubated with primary antibodies. The membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibody. The signal was visualized using an enhanced chemiluminescence (Amersham, Buckinghamshire, UK).

Cell migration and Matrigel invasion assay

Gastric cancer cells were harvested with 0.05% trypsin containing 0.02% EDTA (Sigma-Aldrich), and suspended in RPMI at a concentration of 3 × 10^5 cells/well. Membrane filters (pore size: 8 μm) in disposable 96-well chemotaxis chambers (Neuro Probe, Gaithersburg, MD) were pre-coated for 4 h with 5 mg/ml fibronectin at room temperature. Aliquots (50 μl/well) of the cell suspension were loaded into the upper chambers, and 1% FBS was loaded into the lower chamber. After 24-h incubation, non-migrating cells were removed from the upper chamber with a cotton swab; cells present on the lower surface of the insert were stained with Hoechst33342 (Sigma-Aldrich). Invasive cells were counted under a fluorescence microscope at ×10 magnification.

For the Matrigel invasion assay, 3 × 10^4 cells/well were seeded in the upper chamber, which was coated with Matrigel (5 mg/ml in cold medium, BD Transduction Laboratories, Franklin Lakes, NJ, USA), and serum-free medium containing 1% FBS or control vehicle was added.
to the lower chamber. After 24-h incubation, non-migrating cells were removed from the upper chamber with a cotton swab, and cells present on the lower surface of the insert were stained with Hoechst33342 (Sigma-Aldrich). Invasive cells were then counted under a fluorescence microscope at ×10 magnification.

**Tissue microarrays, immunohistochemistry, and interpretation of results**

A semi-automated tissue arrayer (Beecher Instruments, WI, USA) was used to construct the tissue microarrays. We obtained 3 tissue cores, each 0.6 mm in diameter, from tumor blocks taken from GC patients. Cores were not collected from the more invasive frontal or central areas of the tumors. Slides were baked at 60°C for 30 min, deparaffinized with xylene, and then rehydrated. The sections were subsequently submerged in citrate antigen retrieval buffer, microwaved for antigen retrieval, treated with 3% hydrogen peroxide in methanol to quench endogenous peroxidase activity, and then incubated with 1% bovine serum albumin to block nonspecific binding. Thereafter, the sections were incubated with rabbit anti-Snail (Abcam, UK) overnight at 4°C. Normal rabbit serum was used as a negative control. After washing, tissue sections were treated with secondary antibody, counterstained with hematoxylin, dehydrated, and mounted. At least 500 tumor cells were counted. The percentage of cells with Snail+ nuclei was expressed relative to the total number of tumor cells counted. Nuclear expression of Snail was graded by classifying the extent of positive nuclear staining as ≤50%, 50–75%, or ≥75%.

**Clinicopathological and survival analysis of gastric cancer patients**

We studied a cohort of 314 GC patients who each underwent a gastrostomy with lymph node dissection at Pusan National University Hospital (PNUH) between 2005 and 2007. The group comprised 218 men and 96 women with a mean age of 58.3 years (range, 25–83 years). Standard formalin-fixed and paraffin-embedded sections were obtained from the Department of Pathology, PNUH, and the National Biobank of Korea, PNUH. The study was approved by the Institutional Review Board. None of the patients received preoperative radiotherapy and/or chemotherapy. Adjuvant chemotherapy based on 5-FU was administered on patients with stages II, III and IV after curative resection. We assessed several clinicopathological factors according to the Korean Standardized Pathology Report for Gastric Cancer, the Japanese Classification of Gastric Carcinoma (3rd English edition), and the American Joint Committee on Cancer Staging Manual (7th edition), including tumor site, gross appearance and size, depth of invasion, histological classification (i.e., intestinal or diffuse), and lymphovascular invasion [17-19]. Clinical outcome for each patient was followed from the date of surgery to the date of death or March 1, 2012. Follow-up periods ranged from approximately 1 to 81.5 months (average, 51.4 months). Cases lost to follow-up or death from any cause other than gastric cancer were censored from the survival rate analysis. Clinicopathological features were analyzed using Student’s t-test, the χ² test, or Fisher’s exact test to test for differences in Snail expression. Cumulative survival plots were obtained using the Kaplan–Meier method, and significance was compared using the log-rank test. Prognostic factors were identified using the Cox regression stepwise method (proportional hazard model), adjusted for the patients’ age, gender, tumor site, morphologic type (intestinal versus diffuse). Statistical significance was set at P < 0.05. Statistical calculations were performed with SPSS version 10.0 for Windows (SPSS Inc., Chicago, IL, USA).

**cDNA microarray analysis of GC tissues based on Snail overexpression**

A total of 45 fresh GC tissues were obtained from the National Biobank of Korea, PNUH, and CNUH; approval was obtained from their institutional review boards. Total RNA was extracted from the fresh-frozen tissues using a mirVana RNA Isolation kit (Ambion Inc., Austin, TX). Five hundred nanograms of total RNA was used for cDNA synthesis, followed by an amplification/labeling step (in vitro transcription) using the Illumina TotalPrep RNA Amplification kit (Ambion) to synthesize biotin-labeled cRNA. cRNA concentrations were measured by the RiboGreen method (Quant-iT RiboGreen RNA assay kit; Invitrogen-Molecular Probes, ON, Canada) using a Victor3 spectrophotometer (PerkinElmer, CT), and cRNA quality was determined on a 1% agarose gel. Labeled, amplified material (1500 ng per array) was hybridized to Illumina HumanHT-12 BeadChips v4.0, according to manufacturer’s instructions (Illumina, San Diego, CA). Array signals were developed by streptavidin-Cy3. Arrays were scanned with an Illumina iScan system. The microarray data were normalized using the quantile normalization method in Illumina BeadStudio software. The expression level of each gene was transformed into a log² base before further analysis. Excel was primarily used for statistical analyses. Gene expression differences were considered statistically significant if P < 0.05; all tests were 2-tailed. Cluster analyses were performed using Cluster and Treeview [20]. The gene ontology (GO) program (http://david.abcc.ncifcrf.gov/) was used to categorize genes into subgroups based on biological function. Fisher’s exact test was used to determine whether the proportions of genes in each category differed by group. GC tissues were further
Figure 1 (See legend on next page.)
divided into those with higher (≥75%) and lower (<75%) levels of Snail expression; differential gene expression between the groups was identified. Primary microarray data are available in NCBI’s GEO (Gene Expression Omnibus) database (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE38024).

Results

Regulation of migration and invasion of gastric cancer cells by Snail

Lentiviral-based RNA knockdown and overexpression approaches were used to determine Snail’s role in invasion and migration of gastric cancer cell lines. SNU216 and

Figure 1 Role of Snail in invasion and migration of gastric cancer cell lines. A. SNU216 (upper panel) and SNU484 (lower panel) cells were infected with lentiviruses expressing either non-target shRNA (shNT) or Snail shRNA on day 0, and then harvested on day 7 post-infection. Snail knockdown was determined by RT-PCR and western blotting; stable cell lines were generated for each of the cell lines (sh-Snail). Silencing of Snail in SNU216 and SNU484 cells induced decreased migration and invasion. B. SNU216 (upper panel) and SNU484 (lower panel) cells were infected with lentiviruses expressing either a lentiviral PLKO vector targeting Snail or an empty PLKO vector (EV) on day 0, and then harvested on day 7 post-infection. The overexpression of Snail was determined by RT-PCR and western blotting; stable cell line was generated for each of the cell lines (O/E-snail). Snail overexpression in SNU216 and SNU484 cells induced increased migration and invasion. C. Snail overexpression induced increased mRNA expression of VEGF and MMP11 in SNU216 and SNU484 cells in real-time RT-PCR analysis. Lower panel indicates representative RT-PCR figures for VEGF, MMP11, Snail, and GAPDH. Data show the mean ± SE of at least 3 independent experiments. * indicates P < 0.05 by Student’s t-test.

Figure 2 Snail expression in gastric adenocarcinoma (GC) tissue samples and Kaplan–Meir plots of overall survival of 314 GC patients. Snail was mostly expressed in nuclei of GC cells (intestinal type (A), and diffuse type (signet ring) cells (B)) included in tissue array specimens. Some reactive fibroblasts also showed Snail nuclear expression (magnification: x400). C. Kaplan–Meier analysis of overall survival of GC patients based on Snail expression. A linear relationship between increased Snail nuclear expression and shorter survival was seen among GC patients (P = 0.023). Log-rank test was used to calculate P values.
SNU484 cells used in this study are established gastric adenocarcinoma cell lines from Korean patients. These cells were infected with a lentivirus expressing either non-target or Snail-targeted shRNAs for silencing. A PLKO lentiviral vector that targeted Snail and an empty PLKO vector were used to induce Snail overexpression in SNU216 and SNU484 cells. Polyclonal stable cell lines were selected using puromycin. Snail expression was determined by RT-PCR and western blotting; stable Snail knockdown (sh-Snail) and Snail overexpression cell lines (OE-Snail) were obtained (Figure 1).

To determine Snail's roles in gastric cancer cell invasion, we measured chemotactic invasion by the cells using the Transwell system with filters pre-coated with Matrigel. To measure migration of gastric cancer cells, we assayed cell migration using a Boyden chamber apparatus. Silencing of Snail by shRNA induced decreased migration and invasion of SNU216 and SNU484 cells, as shown in Figure 1A. In contrast to the Snail silencing results, overexpression of Snail induced increased migration and invasion of SNU216 and SNU484 cells, as shown in Figure 1B. Overexpression of Snail was also associated with increased VEGF and MMP11 (Figure 1C).

**Effect of Snail overexpression on tumor aggressiveness and GC patient survival**

Positive nuclear staining for Snail at levels of $\leq 50\%$, 50–75%, and $\geq 75\%$ was observed in 13.4% (42/314), 52.2% (164/314), and 34.4% (108/314), respectively, of the 314 GC patients in immunohistochemical analysis. Snail expression was noted in intestinal and diffuse type of GCs (Figure 2A, B). Snail overexpression ($\geq 75\%$ positivity) significantly correlated with tumor size, gross type, depth of invasion, lymphovascular invasion, perineural invasion, and lymph node metastasis (Table 1). Snail overexpression was also associated with increased tumor size ($P = 0.028$) and excavated gross type ($P < 0.001$); and increased tumor invasiveness, i.e., higher T stage ($P < 0.001$) and the presence of perineural invasion ($P < 0.001$) and lymphovascular tumor emboli ($P = 0.002$). Increased lymph node metastasis was also related to Snail overexpression ($P = 0.002$). In accordance with the above data showing the positive relationship between Snail overexpression and GC aggressiveness, Snail overexpression significantly correlated with overall survival

| Table 1 Relationship between Snail expression and clinicopathological characteristics in 314 patients with gastric cancer |
|---------------------------------------------------------------|
| **Number of patients (N = 314)** | Snail Positivity | $P$ value |
|----------------------------------|-----------------|-----------|
|                                  | $<75\%$ | $\geq75\%$ |
| Age (years)                      | 58.5 ± 10.6 | 59.1 ± 11.9 | 0.695 |
| Sex                              |         |           |         |
| Male                             | 218     | 143       | 75      | 0.996 |
| Female                           | 96      | 63        | 33      |         |
| Tumor size                       |         |           |         |
| $\leq$4.0 cm                     | 192     | 135       | 57      | 0.028 |
| $>$4.0 cm                        | 122     | 71        | 51      |         |
| Location                         |         |           |         |
| Upper/Middle                     | 167     | 112       | 55      | 0.561 |
| Lower                            | 147     | 94        | 53      |         |
| Invasion depth                   |         |           |         |
| T1                               | 160     | 127       | 33      | $<0.001$ |
| T2                               | 41      | 26        | 15      |         |
| T3                               | 68      | 33        | 35      |         |
| T4                               | 43      | 19        | 24      |         |
| Gross type                       |         |           |         |
| Elevated                        | 77      | 51        | 26      | $<0.001$ |
| Flat/depressed                   | 131     | 105       | 26      |         |
| Excavated                       | 106     | 50        | 56      |         |
| Histological type                |         |           |         |
| Intestinal                      | 182     | 123       | 59      | 0.609 |
| Diffuse                         | 122     | 76        | 46      |         |
| Mixed                            | 10      | 7         | 3       |         |
| Perineural invasion              |         |           |         |
| Negative                        | 202     | 150       | 52      | $<0.001$ |
| Positive                        | 111     | 55        | 56      |         |
| Lymphovascular emboli            |         |           |         |
| Negative                        | 193     | 139       | 54      | 0.002 |
| Positive                        | 120     | 66        | 54      |         |
| Lymph node metastasis            |         |           |         |
| N0, N1                          | 270     | 186       | 84      | 0.002 |
| N2, N3                          | 44      | 20        | 24      |         |

| Table 2 Multivariate survival analysis with Cox regression model in 314 gastric cancers |
|-----------------------------------------------|
| **Variables** | **B** | **SE** | **HR (95% CI)** | **P** |
| Age ($\leq$59 versus > 59)                    | -0.438 | 0.264 | 0.645 (0.385-1.082) | 0.097 |
| Gender (male versus female)                   | -0.037 | 0.267 | 0.963 (0.571-1.626) | 0.889 |
| Site (upper and middle versus lower)          | 0.635 | 0.264 | 1.887 (1.126-3.316) | 0.016 |
| Lauren (intestinal vs diffuse)                 | -0.537 | 0.263 | 0.585 (0.349-0.978) | 0.041 |
| Snail ($\geq 75\%$ versus <75%)               | -0.528 | 0.248 | 0.590 (0.363-0.958) | 0.033 |

Note: B, coefficient; HR, hazard ratio; CI, confidence interval.
### Table 3 Genes differentially expressed in GC specimens with higher levels of Snail expression

| PROBE_ID     | SYMBOL | NAME                                                                 |
|--------------|--------|----------------------------------------------------------------------|
| ILMN_2374449 | SPP1   | Secreted phosphoprotein 1                                           |
| ILMN_2337923 | TPDS2L1| Tumor protein DS2-like 1                                             |
| ILMN_1679838 | WBPS   | WW domain binding protein 5                                           |
| ILMN_2078592 | C6orf105| Androgen-dependent TFPI-regulating protein                           |
| ILMN_1714383 | TPDS2L1| Tumor protein DS2-like 1                                             |
| ILMN_1674817 | C1orf115| Chromosome 1 open reading frame 115                                  |
| ILMN_1813561 | SCIN   | Scinderin                                                            |
| ILMN_1759818 | SORL1  | Sortilin-related receptor, L(DLR class) A repeats containing         |
| ILMN_1745686 | MFHAS1 | Malignant fibrous histiocytoma amplified sequence 1                   |
| ILMN_2060115 | SORL1  | Sortilin-related receptor, L(DLR class) A repeats containing         |
| ILMN_2337263 | PKB    | Protein kinase (cAMP-dependent, catalytic) inhibitor beta             |
| ILMN_2173835 | FTHL3  | Ferritin, heavy polypeptide 1 pseudogene 3                           |
| ILMN_1791057 | IFNAR2 | Interferon (alpha, beta and omega) receptor 2                        |
| ILMN_1807114 | LOC255620 | Similar to unc-93 homolog B1 (C. elegans), transcript variant 1 (LOC255620), mRNA |
| ILMN_1669393 | GGT1   | Gamma-glutamyltransferase 1                                          |
| ILMN_1685798 | MAGEA6 | Melanoma antigen family A, 6                                        |
| ILMN_3269395 | GGT2   | Gamma-glutamyltransferase 2                                          |
| ILMN_1669833 | SH2B2  | SH2B adaptor protein 2                                               |
| ILMN_3238534 | LOC100133817 | Hypothetical protein LOC100133817                                   |
| ILMN_2099315 | TRPM8  | Transient receptor potential cation channel, subfamily M, member 8   |
| ILMN_3298065 | LOC29195| Similar to apical early endosomal glycoprotein                       |
| ILMN_1717726 | FLJ43752| Long intergenic non-protein coding RNA 336                           |
| ILMN_1670452 | ANKR0220A1| Ankyrin repeat domain 20 family, member A1                          |
| ILMN_3201060 | LOC100132655| Hypothetical protein LOC100132655                                   |
| ILMN_3282829 | LOC22283| Similar to iduronate 2-sulfatase (Hunter syndrome)                   |
| ILMN_2339691 | SIVN1  | Synovial apoptosis inhibitor 1, synoviolin                          |
| ILMN_1798549 | SLC30A2| Solute carrier family 30 (zinc transporter), member 2               |
| ILMN_3191898 | LOC100129630| Hypothetical LOC100129630                                           |
| ILMN_1704204 | LOC42204| Ankyrin repeat domain-containing protein 26-like                     |
| ILMN_1682280 | LOC47753| Hypothetical protein LOC47753                                       |
| ILMN_3201944 | LOC46438| Hypothetical LOC46438                                               |
| ILMN_2233314 | SPANX1 | Sperm protein associated with the nucleus, X-linked, family member A1|
| ILMN_3305980 | NS3BP  | NS3BP                                                                |
| ILMN_1747850 | CRM2   | Kielin/chordin-like protein                                          |
| ILMN_1700590 | LOC45590| Similar to cAMP-dependent protein kinase type I-beta regulatory subunit|
| ILMN_1763616 | FLJ32679| Golgin-like hypothetical protein LOC440321                           |
| ILMN_180741  | Hs.552561| Pancreatic islet cDNA clone hbt09590 3, mRNA sequence               |
| ILMN_3302855 | MIR33A | MicroRNA 33a                                                        |
| ILMN_1815716 | LMLN   | Leishmanolysin-like (metallopeptidase M8 family)                     |
| ILMN_1654945 | DNMT3A | DNA (cytosine-5)-methyltransferase 3 alpha                           |
| ILMN_2256050 | SERPINA1| Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1 |
| ILMN_1759487 | EGFLAM | EGF-like, fibronectin type III and laminin G domains                 |
| ILMN_1760410 | LOC653086| Similar to RAN-binding protein 2-like 1 isofrom 2                   |
Table 3 Genes differentially expressed in GC specimens with higher levels of Snail expression (Continued)

| Gene ID       | Description                                                                                       |
|---------------|--------------------------------------------------------------------------------------------------|
| ILMN_1668969  | Mix paired-like homeobox                                                                           |
| ILMN_3279757  | Hypothetical protein LOC100132532                                                                 |
| ILMN_1715372  | Calcium/calmodulin-dependent protein kinase 1, alpha                                               |
| ILMN_1731370  | Chromosome 9 open reading frame 84                                                                |
| ILMN_1679049  | Collectin sub-family member 12                                                                    |
| ILMN_1676011  | Similar to FXYD domain-containing ion transport regulator 6                                        |
| ILMN_1815442  | Similar to Protein KIAA0685                                                                        |
| ILMN_1737213  | Golgin A6 family, member C                                                                         |
| ILMN_1793529  | Myosin                                                                                           |
| ILMN_1709319  | Methyltransferase like 21C                                                                        |
| ILMN_2284930  | Proline rich 20A                                                                                  |
| ILMN_1678310  | Thioredoxinreductase 3 neighbor                                                                   |
| ILMN_1806052  | unc-119 homolog (C. elegans)                                                                      |
| ILMN_2242345  | Lipoprotein, Lp(a)-like 2, pseudogene                                                               |
| ILMN_1687725  | ATPase family, AAA domain containing 5                                                              |
| ILMN_1886395  | Soares_multiple_sclerosis_2NhMSP Homo sapiens cDNA clone IMAGp998G11618; IMAGE:126826, mRNA sequence |
| ILMN_3308612  | MicroRNA 149                                                                                      |
| ILMN_1811103  | Protocadherin gamma subfamily B, 5                                                                  |
| ILMN_1736104  | Hypothetical LOC645218                                                                             |
| ILMN_1824307  | Full-length cDNA clone CS0DF20YK03 of Fetal brain of Homo sapiens                                   |
| ILMN_1803871  | Rhodopsin                                                                                         |
| ILMN_3237134  | Similar to butyrate-induced transcript 1                                                           |
| ILMN_1714191  | Similar to Y46GSA.1a                                                                               |
| ILMN_3246580  | e3 ubiquitin-protein ligase UBRS-like                                                               |
| ILMN_3229028  | hCG1981531                                                                                        |
| ILMN_3239734  | Uncharacterized LOC100134822                                                                     |
| ILMN_1769785  | SH3 domain containing ring finger 3                                                                 |
| ILMN_3309864  | MicroRNA 449b                                                                                     |
| ILMN_1653927  | small nucleolar RNA, C/D box 83A                                                                   |
| ILMN_3206648  | uncharacterized LOC151174                                                                         |
| ILMN_1652023  | ArfGAP with FG repeats 2                                                                            |
| ILMN_1749776  | Similar to hypothetical protein LOC284701                                                            |
| ILMN_1671985  | Hypothetical protein LOC646829                                                                     |
| ILMN_1684499  | Similar to deubiquitinating enzyme 3                                                                |
| ILMN_1676452  | ADAM metallopeptidase with thrombospondin type 1 motif 14                                         |
| ILMN_1723855  | Similar to TBP-associated factor 15 isoform 1                                                        |
| ILMN_1658019  | Hypothetical protein LOC648447                                                                     |
| ILMN_3227091  | Hypothetical LOC278701                                                                             |
| ILMN_1767469  | Hypothetical protein LOC650781                                                                     |

Genes downregulated in specimens with higher levels (>75%) of Snail expression (P< 0.05)

| Gene ID       | Description                                                                                       |
|---------------|--------------------------------------------------------------------------------------------------|
| ILMN_1796466  | Allantocase                                                                                       |
| ILMN_3248008  | LOC442308                                                                                         |
| ILMN_3230623  | Tubulin, beta class I pseudogene                                                                 |
| ILMN_1675961  | Hypothetical protein LOC642263                                                                     |
| ILMN_3165745  | Synthetic construct clone NISTag41 external RNA control sequence                                   |

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| Gene ID       | Description                                                                 |
|--------------|-----------------------------------------------------------------------------|
| ILMN_324240  | HCG8, Similar to Tripartite motif protein 44 (DIPB protein) (Mc7 protein)    |
| ILMN_324733  | LOC100131898 Hypothetical protein LOC100131898                              |
| ILMN_3195376 | LOC100130092 Similar to MAPRE1 protein                                          |
| ILMN_2123683 | FLJ43763 Uncharacterized LOC642316                                           |
| ILMN_1730601 | FAM119A, Family with sequence similarity 194, member A                         |
| ILMN_1652015 | LOC647451 Similar to heat shock protein 90Bf                                   |
| ILMN_1784349 | LOC647191 Similar to Kinase suppressor of ras-1 (Kinase suppressor of ras) (mKSR1) (Hb protein) |
| ILMN_3251375 | WBP11P1, WW domain binding protein 11 pseudogene 1                             |
| ILMN_1911713 | Hs.550068 Homosapiens clone UI-E-EJ1-ajn-i-16-O-UI1, mRNA sequence            |
| ILMN_1888057 | LOC649397 Similar to Tripartite motif protein 44 (DIPB protein) (Mc7 protein) |
| ILMN_3229818 | LOC729828, mRNA sequence                                                      |
| ILMN_1654987 | HCG2P7, Similar to HLA complex group 2 pseudogene 7                             |
| ILMN_1683453 | RTBDN Retbindin                                                                  |
| ILMN_1804993 | LOC653786, Otoancorin pseudogene                                               |
| ILMN_1914891 | Hs.334272, Athersys RAGE Library homo sapiens DNA, mRNA sequence               |
| ILMN_322536  | LOC100129315, LOC100129315, mRNA sequence                                     |
| ILMN_3203388 | LOC100130855 Hypothetical protein LOC100130855 LOC100130855, mRNA sequence   |
| ILMN_1656553 | LOC653160, transcript variant LOC653160, mRNA sequence                        |
| ILMN_1709035 | HAS2, Hyaluronan synthase 2                                                   |
| ILMN_1733783 | LOC652790, Similar to anaphase promoting complex subunit 1                    |
| ILMN_2209221 | DMRT1, Doublesex and mab-3 related transcription factor 1                    |
| ILMN_1815118 | ZNF554, Zinc finger protein 554                                               |
| ILMN_3293210 | LOC100131031 Similar to hCG20411190 LOC100131031, mRNA sequence              |
| ILMN_1703222 | FRS2, Fibroblast growth factor receptor substrate 2                           |
| ILMN_1732807 | GPRC6A, G protein-coupled receptor family C, group 6, member A               |
| ILMN_1875332 | Hs.545227, he15g04.x1 NCI_CGLP_U11 Homo sapiens DNA clone IMAGE:2262783 3 similar to contains PTR5b2 PTR5 repetitive element, mRNA sequence |
| ILMN_3235789 | BPY2C, Basic charge, Y-linked, 2C                                              |
| ILMN_3203116 | LOC100131967, Misc_RNA LOC100131967, mRNA sequence                           |
| ILMN_2198802 | FAM22G, Family with sequence similarity 22, member G                          |
| ILMN_1858700 | Hs.538588, Zo20c06s1 Soares testis_NHT homo sapiens DNA clone IMAGE:412618 3, mRNA sequence |
| ILMN_1873107 | LOC100131031 Similar to hCG20411190 LOC100131031, mRNA sequence              |
| ILMN_1891673 | Hs.164254, Hb73c02.x1 NCI_CGAP_U11 Homo sapiens DNA clone IMAGE:288834 3, mRNA sequence |
| ILMN_3006632 | LOC643802, u3 small nucleolarribonucleoprotein protein MPP10-like             |
| ILMN_1883034 | Hs.546089, RST29145 Athersys RAGE Library homo sapiens DNA, mRNA sequence    |
| ILMN_2373335 | LG3, Ligase III, DNA, ATP-dependent                                            |
| ILMN_3239639 | CD200R1L, CD200 receptor 1-like                                               |
| ILMN_1870857 | Hs.148168, Barstead spleen HPLRB2 Homo sapiens DNA clone IMAGE:998L113601 ; IMAGE:1425178, mRNA sequence |
| GenBank Accession | Description                                                                 |
|-------------------|------------------------------------------------------------------------------|
| ILMN_1813909      | CRSP2 Mediator complex subunit 14                                             |
| ILMN_1891885      | Hs.332843 Soares_NFL_T_GBC_S1 Homo sapiens cDNA clone IMAGE:1841748, mRNA sequence |
| ILMN_3253126      | LOC100133558 Similar to LOC100133558, mRNA sequence                           |
| ILMN_1677186      | MGC52498 Family with sequence similarity 159, member A                      |
| ILMN_3252608      | HCRP1 Hepatocellular carcinoma-related HCRP1                                  |
| ILMN_1652871      | PS1CRS Phospholipid scramblase family, member 5                               |
| ILMN_1698894      | OR5AS1 Olfactory receptor, family 5, subfamily AS, member 1                  |
| ILMN_1705828      | RICTOR RPTOR independent companion of MTOR, complex 2                         |
| ILMN_1683046      | OR6Y1 Olfactory receptor, family 6, subfamily Y, member 1                     |
| ILMN_2114812      | ONECUT1 One cut homeobox 1                                                   |
| ILMN_1770248      | PDLIM2 CDZ and LIM domain 2 (mystique)                                       |
| ILMN_1784272      | CD1E CD1e molecule                                                           |
| ILMN_1755635      | FLJ33534 Hypothetical protein FLJ33534 (FLJ33534), mRNA sequence             |
| ILMN_1790676      | TRY1 Protease, serine, 1 (trypsin 1)                                          |
| ILMN_1693448      | LOC643811 Similar to FERM domain containing 6                                |
| ILMN_1865604      | Hs.547267 soares_NFL_T_GBC_S1 Homo sapiens cDNA clone IMAGE:4800534 5, mRNA sequence |
| ILMN_3308698      | MIR1276 MicroRNA 1276                                                        |
| ILMN_1714014      | LOC644401 NMDA receptor regulated 2 pseudogene                                |
| ILMN_2114815      | C1orf104 RUSC1 antisense RNA 1 (non-protein coding)                          |
| ILMN_191044       | Hs.540915 nf66b6s1 NCI_CGAP_Co3 Homo sapiens cDNA clone IMAGE:924851 3, mRNA sequence |
| ILMN_1748543      | TRC Stereocilin                                                               |
| ILMN_1675221      | DGKZ Diacylglycerol kinase, zeta                                              |
| ILMN_1722623      | LOC10035748 Similar to dipeptidylaminopeptidase-like protein 6 (dipeptidylpeptidase VI) (dipeptidylpeptidase 6) (dipeptidylpeptidase-related protein) (DPPX) |
| ILMN_1817113      | HS.547985 UI-H-Bip-abm-h-10-0-U1s1 NCI_CGAP_Sub2 Homo sapiens cDNA clone IMAGE:2712450 3, mRNA sequence |
| ILMN_1793525      | KIR2DS3 Killer cell immunoglobulin-like receptor, two domains, short cytoplasmic tail, 3 |
| ILMN_2451651      | C10orf172 V-set and transmembrane domain containing 4                        |
| ILMN_1746277      | MLLT4 Myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 4 |
| ILMN_1678246      | LOC644001 Hypothetical protein LOC644001                                     |
| ILMN_3257856      | LOC100130938 Hypothetical LOC100130938 (LOC100130938), mRNA sequence         |
| ILMN_1865630      | Hs.163333 Soares_testis_NHT_Homo sapiens cDNA clone IMAPg998A031828, mRNA sequence |
| ILMN_2152028      | LOC642452 Hypothetical LOC642452 (LOC642452), mRNA                             |
| ILMN_3245479      | LOC649330 Heterogeneous nuclear ribonucleoprotein C-like                      |
| ILMN_1905832      | HS.564127 UI-E-DW1-ahc-g-05-0-U1s1 UI-E-DW1 Homo sapiens cDNA clone IMAGE:2712450 3, mRNA sequence |
| ILMN_1897251      | HS.547715 UI-E-EIo-ahv-e-11-0-U1s1 UI-E-EIO Homo sapiens cDNA clone IMAGE:265999 3, mRNA sequence |
| ILMN_1782800      | LOC651410 Hypothetical protein LOC651410                                      |
| ILMN_1732554      | ZNF346 Zinc finger protein 346                                                |
| ILMN_1674014      | LOC653878 Similar to Cytosolic acyl coenzyme A thioester hydrolase, inducible (Long chain acyl-CoA thioester hydrolase) (Long chain acyl-CoA hydrolase) (CTE-I) (CTE-Ib) |
| ILMN_1911501      | HS.543805 xi8908x1 NCI_CGAP_MelB Homo sapiens cDNA clone IMAGE:265999 3, mRNA sequence |
| ILMN_1878305      | HS.262789 x07d09x1 NCI_CGAP_Co20 Homo sapiens cDNA clone IMAGE:2666033 3, mRNA sequence |
| ILMN_1858245      | HS.156566 Soares_testis_NHT_Homo sapiens cDNA clone IMAPg998M073519, mRNA sequence |
| ILMN_1704313      | GSTCD Glutathione S-transferase, C-terminal domain containing                |
| ILMN_1707398      | ESRB Estrogen-related receptor beta                                           |
| ILMN_3307954      | L3MBTL4 l(3)mbt-like 4 (Drosophila)                                          |
among GC patients ($P = 0.023$) (Figure 2C). A linear relationship was observed between increased nuclear expression of Snail and shortened survival ($≤ 50\%: 76.6 ± 2.7$ months; $50–75\%: 68.5 ± 2.0$ months; $≥ 75\%: 63.3 ± 2.8$ months). Snail overexpression ($≥ 75\%$ positivity) was identified as an independent predictor of poor prognosis in 314 patients with GC, adjusted for age, sex, histologic classification, and tumor location, using a Cox regression proportional hazard model ($P = 0.033$; Table 2).

Identification of gene expression patterns based on Snail overexpression using cDNA microarrays

cDNA microarrays were used to compare gene expression profiles of 45 GC specimens. We identified 213 genes differentially expressed in GC specimens with higher levels of Snail expression (Continued)

| GenBank Accession | Description                        | Source                  |
|-------------------|------------------------------------|-------------------------|
| ILMN_1851244      | UI_H_B11_aex-h-12-O-US31          | Homo sapiens cDNA clone IMAGE:2720903 3, mRNA |
| ILMN_1828556      | nac23e12x1                      | Homo sapiens cDNA clone IMAGE:3394270 3, mRNA sequence |
| ILMN_1692894      | LOC654042             | Similar to dehydrogenase/reductase (SDR family) member 4 like 2 |
| ILMN_1893728      | Hs.377660            | Homo sapiens cDNA FLJ26242 fis, clone DMC00770 |
| ILMN_1667005      | LOC652676             | Similar to similar to hypothetical protein FLJ36144 |
| ILMN_3241607      | LOC100132106         | Hypothetical LOC100132106 |
| ILMN_1797503      | GOLGA8G               | Golgin A8 family, member G |
| ILMN_1828034      | Hs.154513              | Human insulinoma Homo sapiens cDNA clone IMAGE6027645 3, mRNA sequence |
| ILMN_1886816      | Hs.544491              | Soraes_NHMPu_51 Homo sapiens cDNA clone IMAGE:1934100 3, mRNA sequence |
| ILMN_1847950      | Hs.505938              | NCI_CGAP_GC6 Homo sapiens cDNA clone IMAGE:2479010 3, mRNA sequence |
| ILMN_1734479      | ACCN3                     | Acid-sensing (proton-gated) ion channel 3 |
| ILMN_1675025      | H2BFM                    | H2B histone family, member M |
| ILMN_2073279      | SIM1                     | Single-minded homolog 1 (Drosophila) |
| ILMN_1910185      | Hs.98563                | zv57/h30.3s1 Soares_total_fetus_Nb2HF8_9w Homo sapiens cDNA clone IMAGE:774197 3, mRNA sequence |
| ILMN_3251491      | UQCR8                    | Ubiquinol-cytochrome c reductase binding protein |
| ILMN_2180315      | ATG4D                    | ATG4 autophagy related 4 homolog D (S. cerevisiae) |
| ILMN_1885583      | Hs.542934              | Homo sapiens cDNA FLJ26431 fis, clone DMC00730 |
| ILMN_1743301      | MSR1                     | Macrophage scavenger receptor 1 |
| ILMN_1809820      | LOC648963              | Similar to retinitis pigmentosa 1-like 1 |
| ILMN_1869348      | Hs.460114               | UI-E-EJ0-ahv-d-07-O-UI1 Homo sapiens cDNA clone UI-E-EJ0-ahv-d-07-O-UI 3, mRNA sequence |
| ILMN_1711332      | TFEC                     | Transcription factor EC |
| ILMN_2228338      | IRAK1BP1                | Interleukin-1 receptor-associated kinase 1 binding protein 1 |
| ILMN_1756455      | ILSRA                    | Interleukin 5 receptor, alpha |
| ILMN_1719202      | ZNF174                  | Zinc finger protein 174 |
| ILMN_1847029      | Hs.553290               | HESC3_84_D06.g1_A036 Human embryonic stem cells Homo sapiens cDNA clone IMAGE:748345 5, mRNA sequence |
| ILMN_1740217      | HACE1                   | HECT domain and ankyrin repeat containing 3 ubiquitin protein ligase 1 |
| ILMN_1787464      | LOC651296               | Similar to RAB, member of RAS oncogene family-like 2B isoform 1 |
| ILMN_1734096      | DCLRE1A                  | DNA cross-link repair 1A |
| ILMN_2391333      | CYP20A1                  | Cytochrome P450, family 20, subfamily A, polypeptide 1 |
| ILMN_2226314      | DBR1                     | Debranching enzyme homolog 1 (S. cerevisiae) |
| ILMN_2379560      | CDC14B                  | CDC14 cell division cycle 14 homolog B (S. cerevisiae) |
| ILMN_2078466      | DZIP1L                   | DAZ interacting protein 1-like |
| ILMN_1653039      | LOC642934               | Hypothetical protein LOC642934 (LOC642934), mRNA |
| ILMN_2044293      | KBTBD7                   | Kelch repeat and BTB (POZ) domain containing 7 |
| ILMN_1809951      | ZNF200                  | Zinc finger protein 200 |
| ILMN_1760280      | NXT1                     | NTF2-like export factor 1 |
| ILMN_1657796      | STMIN1                   | Stathmin 1 |
| ILMN_1793578      | ZFP37                    | Zinc finger protein 37 homolog (mouse) |
Figure 3 Supervised clustering analysis of 45 gastric adenocarcinoma (GC) specimens and 172 genes. Hierarchical clustering was used for 45 GC specimens and 213 genes. Data are shown in a matrix format, with rows representing individual genes and columns representing tissues. Each cell in the matrix represents the expression level of a gene featured in an individual tissue. Red and green cells reflect GCs with higher (≥75%) and lower (<75%) levels of Snail expression, respectively. Matrix patterns for specimens clustered into 2 distinct groups, except for one sample with higher levels of Snail expression.
genes that were differentially expressed at significant levels ($P < 0.05$) between GC specimens with higher ($\geq 75\%$) and lower levels ($<75\%$) of Snail expression (Table 3). Of these 213 genes, 82 were upregulated and 131 were downregulated in the GC specimens with higher levels ($\geq 75\%$) of Snail expression. We used hierarchical clustering analysis to assess the 213 genes and 45 GC specimens; supervised clustering analysis gave patterns for samples with higher and lower levels of Snail expression clustered into 2 distinct groups, except for one sample with higher levels of Snail expression (Figure 3). To investigate the biological functions involved in discriminating genes, we performed a GO category analysis. Eleven genes were associated with involved in discriminating genes, we performed a GO (Figure 3). To investigate the biological functions

Discussion

Snail is reportedly a key regulator of tumor progression and metastasis via increased MMP expression and tumor invasion [26,27]. Similarly, we found that upregulated Snail expression increased gastric cancer cell invasion/migration, whereas downregulated Snail expression decreased gastric cancer cell invasion/migration. Yang et al. reported that Snail overexpression in hepatocellular carcinoma cell lines induced increased invasiveness/metastasis [13]. In addition, Kosaka et al. reported that Snail knockdown was associated with decreased invasive capacity of a urothelial carcinoma cell line, supporting our results [12]. We also found that Snail overexpression induced increased expression of VEGF and MMP11, which are known markers of tumor invasion and metastasis. Jin et al. also reported that Snail knockdown by antisense Snail was associated with inhibited MMP activity, demonstrating the importance of regulating MMP activity in cancer metastasis. Furthermore, Peinado et al. reported that I MDCK cells with Snail overexpression had increased angiogenesis and VEGF [28]. We also observed increased VEGF in gastric cancer cells with Snail overexpression.

The clinical importance of Snail in various carcinomas, including non-small cell lung carcinomas, ovarian carcinomas, urothelial carcinomas, hepatocellular carcinoma, and breast cancer, is well known, as is the poor prognosis associated with Snail overexpression [10-13,29]. However, only limited immunohistochemical data have been available on Snail expression in GC, with no comprehensive clinical and functional analysis of Snail expression in GC patients. Kim et al. reported immunohistochemical data indicating that Snail expression was an independent indicator of prognosis in tissue microarray specimens [14]. Rye et al. reported that the combination of Snail, vimentin, E-cadherin, and CD44 was also significantly associated with poor prognosis in gastric cancer [15]. In contrast, no

Table 4 Cellular functions of selected genes that are differentially expressed in GC specimens that overexpress Snail

| Probe ID | Gene acronym | Gene name | Accession No | $P$ value |
|----------|--------------|-----------|--------------|-----------|
| ILMN_1735987 | EGFLAM | EGF-like, fibronectin type III, and laminin G domains ($\uparrow$) | NM_183280 1 | 0.005 |
| ILMN_2114812 | ONECUT1 | One cut homeobox 1 ($\downarrow$) | NM_004498 | 0.002 |
| ILMN_2374449 | SPP1 | Secreted phosphoprotein 1 ($\uparrow$) | NM_000582 | 0.004 |
| ILMN_1676452 | ADAMTS14 | ADAM metallopeptidase with thrombospondin type 1 motif, 14 ($\uparrow$) | NM_080722 | 0.005 |
| ILMN_1759487 | EGFLAM | EGF-like, fibronectin type III, and laminin G domains ($\uparrow$) | NM_183280 1 | 0.005 |
| ILMN_1683453 | FRAS1 | Fraser syndrome 1 ($\downarrow$) | NM_020875 | 0.003 |
| ILMN_2171057 | IFNAR2 | Interferon (alpha, beta, and omega) receptor 2 ($\uparrow$) | NM_207585 | 0.001 |
| ILMN_1756455 | ILSRA | Interleukin 5 receptor, alpha ($\downarrow$) | NM_000564 | 0.004 |
| ILMN_1747850 | CRIM2 | Kielin/chordin-like protein ($\uparrow$) | NM_199349 | 0.005 |
| ILMN_1743301 | MSRI | macrophage scavenger receptor 1 ($\downarrow$) | NM_002445 | 0.002 |
| ILMN_2374449 | SPP1 | Secreted phosphoprotein 1 ($\uparrow$) | NM_000582 | 0.004 |
| ILMN_2256050 | SERPINA1 | Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1 ($\downarrow$) | NM_000295 | 0.002 |
| ILMN_2060115, ILMN_1759818 | SORL1 | Sortilin-related receptor, L(DLR class) A repeats-containing ($\uparrow$) | NM_0003105 | 0.03 |

NOTE: $\uparrow$, upregulation; $\downarrow$, downregulation.
significant correlation between tumor stage and Snail expression was noted in upper gastrointestinal tract adenocarcinoma, including cancers of the esophagus, cardia, and stomach [30]. In our study, overexpression of Snail (≥75% nuclear Snail expression) was significantly associated with tumor progression, lymph node metastases, lymphovascular invasion, perineural invasion, and poor prognosis in GC patients. Recently, He et al. reported Snail to be an independent prognostic predictor of patient survival among gastric cancer patients; this is in agreement with our data [31]. Although 5-FU based adjuvant chemotherapy for advanced or metastatic gastric adenocarcinoma was usually performed in our cohort, further work is required to reveal exact significance of Snail expression as predictor of chemotherapy response in gastric adenocarcinoma. For the practical use of Snail as a tissue biomarker in predicting lymph node metastasis and poor prognosis, we defined a cut-off value of 75% positive nuclear expression for Snail overexpression. There are wide variations in cut-off values for Snail overexpression in different types of cancer; for example, 75% is used in non-small cell lung carcinoma [11], 100 (score of mean percentage × intensity, range 0–300) is used in urothelial carcinomas [12], and 50% is used in hepatocellular carcinoma [13]. For gastric cancers, cut-off values of 10% [14] and 5% [15] positive nuclear expression of Snail have been reported. Further work is required to determine a practical consensus cut-off value for Snail overexpression.

A total of 213 genes that were differentially expressed among GC samples with higher (≥75%) and lower levels of Snail expression were clustered into 2 distinct groups: those associated with regulation of cancer cell–ECM adhesion, and those associated with ECM protein regulation, such as ONCIL1 [21], ADAMTS [22], IFNAR2 [23], MSRI [24], and SORLI [25]. These functions indicate that Snail greatly affects cancer cell migration and metastasis by regulating attachment of tumor cells to basement membranes, degradation of local connective tissue, and penetration and migration of tumor cells through stroma.

Conclusions

In this study, we showed that Snail overexpression induced increased migration and invasion in gastric cancer cell lines. Snail overexpression was also significantly associated with tumor progression, lymph node metastases, lymphovascular invasion, perineural invasion, and poor prognosis in GC patients. We identified 213 genes that were differentially expressed in GC tissues that overexpressed Snail, including genes related to metastasis and invasion by tumor cells. Our results indicate that Snail is crucial in controlling progression and metastasis of gastric cancer. Thus Snail may be used as a predictive biomarker for evaluating prognosis or aggressiveness of GCs.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

NRS, EHL, CIC and DYP were involved in the design of the study, collected the clinical data, performed the immunohistochemical analysis and drafted the manuscript. HJK performed the analysis of microarray data and helped to draft the manuscript. ISC provided general support and helped to analyze the microarray data. GHK, TYJ, DHK and JHL provided the study materials or patients. DYP supervised the study. All authors read and approved the final manuscript.

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