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Expression of *ADAM12* in Gastric Cancer and its Relation to Tumor Cell Behavior and Prognosis

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Abstract. *Background/Aim: A disintegrin and metalloprotease (ADAM) 12 expression has been found up-regulated in various cancer types. The aim of the study was to evaluate whether ADAM12 affects oncogenic behavior of gastric cancer (GC) cells and investigate its prognostic value. Materials and Methods: The effect of ADAM12 on tumor cell behavior was examined using the small interfering RNA and pcDNA6-myc vector in human GC cell lines. Expression of ADAM12 in GC tissues was confirmed by immunohistochemistry. Apoptosis and proliferation were determined by a terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay and immunohistochemical staining for Ki-67. Results: ADAM12 overexpression enhanced tumor cell migration and invasion in AGS and SNU638 cells. Down-regulation of caspase-3 and PARP activity due to ADAM12 overexpression enhanced tumor cell proliferation and inhibited apoptosis. The expression of Snail and Vimentin increased and that of E-cadherin decreased following ADAM12 overexpression. In contrast, ADAM12 knockdown reversed these effects. ADAM12 overexpression increased the phosphorylation of Akt and GSK-3β. The mean Ki-67 labeling index value of ADAM12-positive tumors was significantly higher compared to that of ADAM12-negative tumors. ADAM12 expression was associated with age, tumor size, cancer stage, depth of invasion, lymph node metastasis, and poor survival. Conclusion: ADAM12 enhances tumor progression by increasing cell mobility, enhancing cell proliferation, and inhibiting apoptosis in GC cells. Also, ADAM12 is associated with adverse clinicopathological features and poor survival. It may be used as a molecular marker for the prediction of clinical outcomes of patients with GC.*

Gastric cancer (GC) is one of the leading causes of cancer-related morbidity and mortality worldwide. Recently, the survival of patients with GC has improved dramatically due to early diagnosis and development of new surgical and multimodal therapies. However, the overall survival rates for patients with advanced stage GC remain low (1-3).

Cancer metastasis represents an advanced stage of cancer and is the major cause of cancer therapeutic failure and related morbidity and mortality. Metastasis is a multistep complex process that includes the inhibition of apoptosis, enhanced proliferation of tumor cells, loss of cell polarity, tissue disorganization, perturbation of cell adhesion, and cancer cell migration and invasion, which are all hallmarks of cancer (4-6). Therefore, advances in our understanding of the molecular mechanisms that contribute to the phenotypic change in favor of cancer metastasis have been useful for identifying biomarkers to predict cancer metastasis in patients with GC.

A disintegrin and metalloproteases (ADAMs) are a family of multidomain membrane-anchored proteins that contain a prodomain, zinc-binding metalloprotease domain, disintegrin domain, cysteine-rich region, epidermal growth factor-like domain, transmembrane helix, and cytoplasmic C-terminal tail (7, 8). To date, approximately 40 mammalian ADAM proteins have been identified and are involved in diverse physiological processes, including cell fate, differentiation, adhesion, proteolysis, migration, and growth. Recently, dysregulation of specific ADAMs was associated with rheumatoid arthritis (9), asthma (10), cardiovascular (11) and Alzheimer's disease (12), and cancers (13). In particular, *ADAM8, ADAM9, ADAM10, ADAM17, ADAM28,* and *ADAM29* are up-regulated and involved in tumor formation and progression in GC (14-19). ADAM12 is a transmembrane and secreted protein of the ADAM family. *ADAM12* expression is increased in many tissues, including bone, cartilage, and muscle tissues, characterized by cell

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fusion or growth and/or repair (20, 21). *ADAM12* has been implicated in the tumor development and progression of various human cancers (22-27). Several studies have shown that *ADAM12* is up-regulated in GC, but the clinical and functional relevance remains to be explored (28-31).

The aim of the current study was to evaluate whether *ADAM12* affects the oncogenic biological behavior of GC cells, the expression of *ADAM12* in GC tissues, and its relationship with clinicopathological features, including survival.

Materials and Methods

Cell culture and gene transfection. The human GC cell lines AGS (CRL-1739) andSNU638 (No.00638) were obtained from the American Type Culture Collection (ATCC, Masassa, VA, USA) and the Korea Cell Line Bank (KCLB, Seoul, Republic of Korea), respectively. Human GC cell lines were maintained in RPMI1640 (Hyclone Laboratories, Loan, UT, USA) supplemented with 10% fetal bovine serum (BSA) and antibiotics. For knockdown of the endogenous *ADAM12* gene, small interfering RNAs (siRNAs) were used. Scramble siRNA (AccuTarget™ negative control siRNA) and specific *ADAM12* siRNA (GACUACAACGGGAAAGCAA-dTdT) were purchased from Bioneer Inc. (Daejeon, Republic of Korea). ADAM12 construction was generated using a pcDNA6-myc-His Mammalian expression vector (Invitrogen, Carlsbad, CA, USA). The ADAM12 siRNAs and constructs were transfected using Lipofectamine® RNAiMAX (Invitrogen) and Lipofectamine® 2000 reagent (Invitrogen) according to the manufacturer's instructions. The transfection efficiency of ADAM12 siRNAs and construct was evaluated using western blotting.

Cell proliferation assay. For the cell viability assay, gene transfected cells were seeded at a density of 5×10^4 cells/ml and then incubated for up to 24 h. Cell proliferation was determined with the EZ-CyTox Cell Viability Assay Kit (Daeil Lab Inc., Seoul, Republic of Korea) using a microplate reader (Infinite M200, Tecan, Männedorf, Switzerland).

Flow cytometric analysis. Cell apoptosis was analyzed using flow cytometry (BD FACSCalibur cytometer, Becton Dickinson, San Jose, CA, USA) with BD Pharmigen™ APC Annexin V/7-amino-actinomycin D (7-AAD) (BD Biosciences, Franklin Lakes, NJ, USA) double staining. Apoptotic cells were subjected to BD Cell Quest® version 3.3 (BD Biosciences) and WinMDI version 2.9 (The Scripps Research Institute, San Diego, CA, USA).

Preparation of cell extracts and western blotting. Protein extracts were lysed in RIPA® reagent (Thermo Fisher Scientific, Waltham, MA, USA) with proteinase and phosphatase inhibitors. The protein concentration of the cell lysate was determined by the BCA™ protein assay (Thermo Fisher Scientific) with BSA as the standard. The proteins were separated on SDS-polyacrylamide gels and electro-transferred onto a PVDF membrane (Millipore, Billerica, MA, USA). Membranes were then blocked in 5% BSA and incubated with specific primary antibodies at 4˚C. The specific band of antibodies was visualized by the luminescent image analyzer LAS-4000 (Fujifilm, Tokyo, Japan) with an enhanced chemiluminescence detection system HRP substrate. The antibodies against cleaved caspase-3 (1:1000, #9664), cleaved poly (ADP-ribose) polymerase (PARP) (1:100, #5625), p21 (1:100, #2947), p57 (1:100, #2557), E-cadherin (1:100, #3195), Snail (1:100, #3879), Vimentin (1:100, #5741), glycogen synthase kinase-3β (GSK3β) (1:100, #9315), phospho-GSK3β (Ser9) (1:1,000, #5558), AKT (1:100, #4685) and phospho-Akt (Ser473) (1:1,000, #4060) were used. These were purchased from Cell Signaling (Danvers, MA, USA). The antibody against ADAM12 (1:1,000, ab28747) was purchased from Abcam (Cambridge, UK). Antibody against GAPDH (1:1,000, sc-25778) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Immunoblots were quantified using Multi-Gauge gel analysis software (ver 3.0, Fujifilm, Tokyo, Japan).

Transwell invasion assay. The invasive ability was calculated by the number of cells that passed through the Corning Transwell® Polycarbonate membrane cell culture inserts (Corning Inc., NY, USA) with 8 μM pores. Transwell insert chambers were coated with 1% gelatin overnight and dried at room temperature. Gene transfected cells were seeded on 2×10^5 viable cells in 0.2% BSA medium on the upper chamber. After incubation for 24 h, invaded cells on the bottom surface of the Transwell were stained with Diff-Quik solution (Sysmex, Kobe, Japan). The invaded cells were photographed using a hemocytometer under light microscope (200× magnification) and the number of invaded cells was counted in 4 selected fields (each 1 mm^2). Data are presented as the mean±SE of the number of cells/field in three independent experiments.

Wound healing assay. Cells were seeded in culture inserts (Ibidi GmbH, Planegg, Germany) and wounds were created by gently removing with tweezers after 24 h of incubation. The floating cells were removed with PBS, then serum-free medium was added to the wells. The wounded gap image was captured at different time points within 24 h. The width of the wound at 100× magnification was evaluated by microscopy (Nikon, Tokyo, Japan). The migration distance was normalized to 1 cm after capturing three random sites.

Patients and tissue samples. Fresh GC tissues and paired normal gastric tissues were collected by gastrointestinal endoscopic biopsy at the Hwasun Chonnam University Hospital (Jeonnam, Republic of Korea). GC tissues were obtained from 212 patients who underwent curative surgical resection for GC at the Chonnam National University Hwasun Hospital (Jeonnam, Republic of Korea) between January 2002 and December 2006. The mean age of enrolled patients was 58.47 years. The mean tumor size was 4.27 cm^3. All samples were routinely fixed with 10% formaldehyde and embedded with paraffin. Tissue blocks were selected by viewing the original pathologic slides and selecting those showing the intersection between normal gastric epithelium and the tumor. The pathologic TNM status of these specimens was evaluated according to the standard criteria for TNM staging of the American Joint Committee on Cancer (30). Clinicopathological parameters at the time of surgery were retrieved from medical records. Overall survival was followed from the time of surgery until December 31, 2018. This study was approved by the Institutional Review Board of the Chonnam National University Hwasun Hospital (IRB No. CNUHH-2021-206).

Quantitative real time-polymerase chain reaction (qRT-PCR). Fresh biopsy tissues were homogenized with the TRIzol reagent (Invitrogen), and total RNA was isolated. cDNA was reverse transcribed from 500 ng of total RNA using MMLV reverse

transcriptase (Promega, Madison, WI, USA). qPCR was carried out with TB® Green Premix EX Taq™ II (Takara, Shiga, Japan) and specific *ADAM12* primers (forward-ACACAATAAGTCCCCTGAG and reverse-CACGGAAACCCACTATXTG) in triplicate. GAPDH (forward-GGGCGATGCTGGCGCTGAGT and reverse-CATCAG CAGAGGG GGCAGAG) was used as an endogenous reference. Data were calculated with the $2^{-\Delta\Delta Ct}$ method.

Immunohistochemical analysis of ADAM12 expression. Tissue sections were subjected to deparaffinization and gradual rehydration. After antigen retrieval, the endogenous peroxidase activity was inhibited with Dako REALTM peroxidase blocking solution (Dako, Carpinteria, CA, USA). Tissues were probed with anti-human ADAM12 primary antibody (1:500, E-AB-16133, Elabscience, Huston, TX, USA) overnight at 4˚C. The samples were treated with the Dako REALTM Envision HRP/DAB detection system (Dako) and counterstained with Mayer hematoxylin (Sigma–Aldrich, St. Louis, MO, USA). Stained tissues were viewed and imaged using a light microscope (Olympus, Tokyo, Japan). The immunostaining score of *ADAM12* expression was assessed independently by two parameters without the clinicopathological information of the patients. The immunostaining intensity was determined with four grades as follows: 0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining. The immunostaining extent was estimated in four grades based on the ratio of the strongly stained area to the total tumor area as follows: 0, none; 1, <10%; 2, 10-50%; and 3, >50%. The total score was obtained by multiplying the intensity and extent of immunostaining. Samples with a total score of ≥6 were termed ADAM12-positive, while those with a total score of <6 were termed ADAM12-negative.

Assessment of tumor cell proliferation and apoptosis. Tumor cell proliferation was determined by immunostaining using anti-human Ki-67 antibody. A distinct nuclear immunoreactivity for Ki-67 was considered positive. The Ki-67 labeling index (KI) was defined as the number of Ki-67-positive nuclei per 1,000 tumor cell nuclei. Quantification of tumor cell apoptosis was assessed using DeadEnd™ Colorimetric-terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) system (Promega) according to the manufacturer's instructions. The apoptotic index (AI) was presented as a number of TUNEL-positive cells per 1000 tumor cell nuclei. Counting was performed on five randomly selected microscopic fields (40× original magnification) per sample by two independent blinded observers.

Statistical analysis. The comparison of *ADAM12* expression in various clinicopathological variables was assessed by partitioning Chi-squared and Fisher's exact tests by the Statistical Package for the Social Sciences software (SPSS/PC+ 20.0, Chicago, IL, USA). Survival curves were created using the Kaplan-Meier method with the log-rank test. Statistical significance of the *in vitro* experiments was determined by Student's *t*-test using the Sigma Plot software (Aspire Software International, Ashburn, VA, USA). Differences were considered significant when $p<0.05$.

Results

Expression of ADAM12 in GC cells. To investigate the expression of *ADAM12* in GC cells, ADAM12 protein expression was examined by western blotting in human GC cell lines AGS and SNU638. *ADAM12* expression was absent and weakly observed in AGS and SNU638, respectively. Therefore, we transfected the *ADAM12* gene using ADAM12-pcDNA6-myc construct and knocked it out using ADAM12 siRNA in AGS and SNU638 cells. ADAM12 protein expression in tested cells showed a significant increase at the protein level by the transfection of the ADAM12-pcDNA6-myc construct and a significant decrease by the transfection of ADAM12 siRNA (Figure 1).

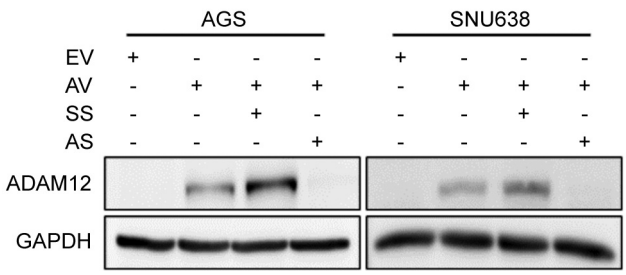

Figure 1. *Expression of A disintegrin and metalloprotease 12 (ADAM12) in gastric cancer (GC) cells. Expression of ADAM12 protein by transfection of the ADAM12-pcDNA6-myc construct (AV) or ADAM12 siRNA (AS) in AGS and SNU638 cells. Western blotting revealed that ADAM12 protein was over-expressed and knocked-down using the AV and AS, respectively. EV, Empty-pcDNA6-myc vector; SS, scramble siRNA.*

Impact of ADAM12 on the migration and invasion of GC cells. The artificial wound gap in pcDNA6-myc-ADAM12-transfected AGS cells became significantly narrower than that in empty-pcDNA6-myc-transfected cells ($p=0.021$ and 0.994, respectively; Figure 2A). *ADAM12* knockdown inhibited *ADAM12*-induced cell migration in AGS and SNU638 cells at 10 h ($p<0.001$and $p<0.001$, respectively; Figure 2A). The number of invading cells significantly increased in pcDNA6-myc-ADAM12-transfected AGS cells compared to that in empty-pcDNA6-myc-transfected cells ($p=0.009$ and $p=0.081$, respectively; Figure 2B). Additionally, *ADAM12* knockdown inhibited ADAM12-induced cell invasion in AGS and SNU638 cells ($p=0.003$ and $p=0.011$, respectively; Figure 2B).

Impact of ADAM12 on tumor cell survival of GC cells. To determine the potential effects of *ADAM12* on cell proliferation, cells were subjected to a cell proliferation assay at 0, 1, and 2 days after transfection with pcDNA6-myc-ADAM12 or ADAM12 siRNA. The number of proliferating cells, as determined by absorbance, increased significantly in the pcDNA6-myc-ADAM12-transfected AGS and SNU638 cells compared to that in empty-pcDNA6-myc-transfected cells ($p=0.023$ and $p=0.014$, respectively; Figure 3). In contrast, ADAM12 siRNA-transfected AGS and SNU638 cells showed significantly decreased proliferation compared to that in scrambled

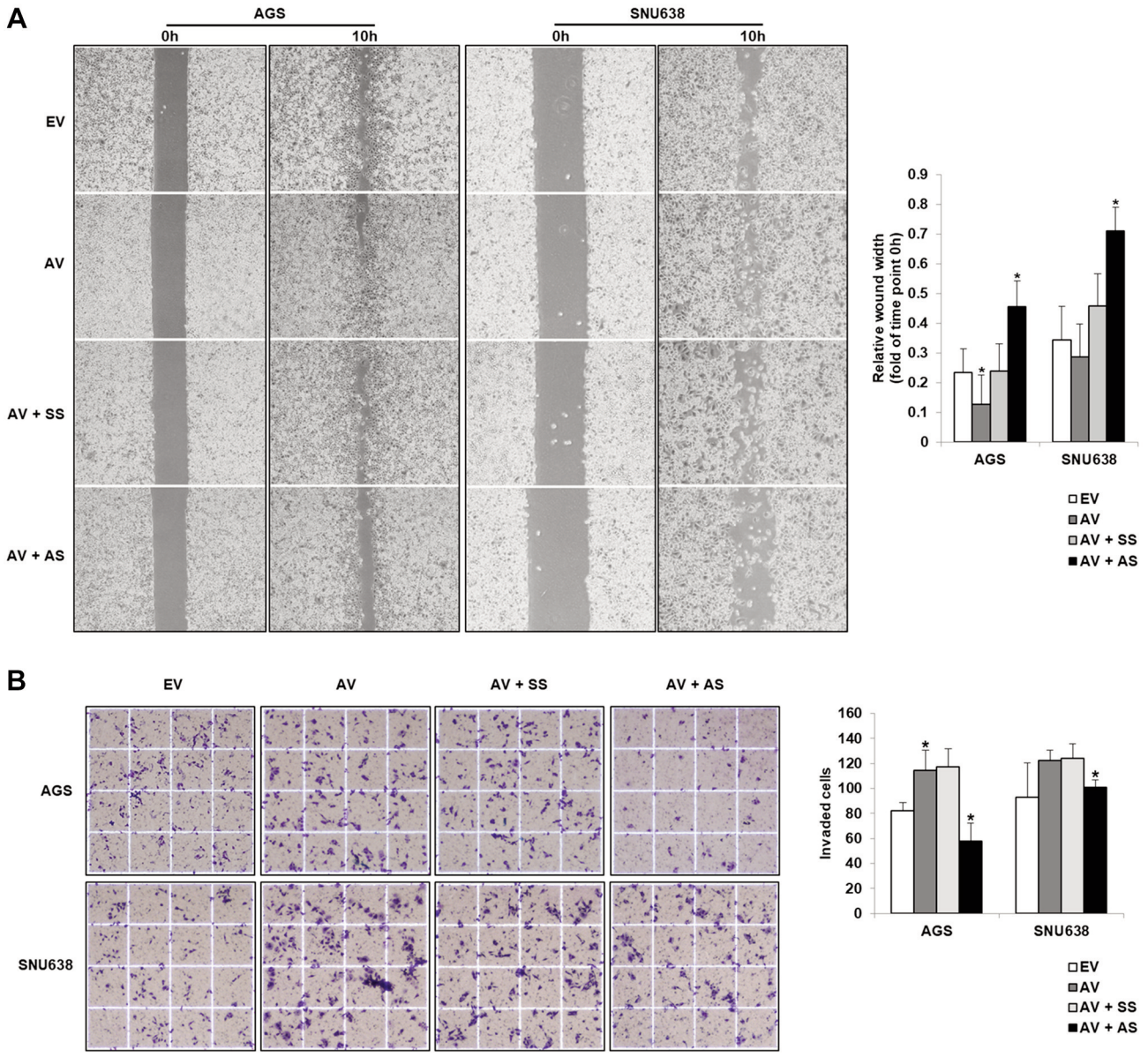

Figure 2. *Impact of A disintegrin and metalloprotease 12 (ADAM12) on the migration and invasion of gastric cancer (GC) cells. (A) Impact of ADAM12 on the migration of GC cells. A wound healing assay using the siRNA-transfected cells was performed and graphs of cell migration are displayed as relative healing distances (mean±SD, n=3; *p<0.05). The artificial wound gap in the plates of scramble siRNA (SS)-transfected cells became significantly narrower than that in AS-transfected cells at 10 h in AGS and SNU638 cells. (B) Impact of ADAM12 in the invasion of GC cells. An invasion assay using the siRNA-transfected cells was performed. Stained invading cells were counted and are represented as a graph between groups. The number of ADAM12 siRNA (AS)-transfected cells that invaded was significantly lower than that in SS-transfected AGS and SNU638 cells (mean±SD, n=3; *p<0.05). EV, Empty-pcDNA6-myc vector; AV, ADAM12-pcDNA6-myc construct; SD, standard deviation.*

siRNA-transfected cells (p=0.035 and p=0.044, respectively; Figure 3). To evaluate the impact of *ADAM12* on apoptosis, we performed flow cytometric analyses. The apoptotic rate decreased significantly in AGS and SNU638 cells after overexpression of *ADAM12* (9.10% *vs*. 5.71% and 18.38% *vs*. 13.90%, respectively). Additionally, the apoptotic rate induced by the transfection of ADAM12 siRNA significantly increased compared to that induced by transfection of the scrambled siRNA (6.38% *vs*. 13.40% and 18.02% *vs*. 20.39%, respectively) in AGS and SNU638 cells (Figure 4A). To determine the activation of caspases during overexpression and knockdown of *ADAM12*, we further investigated caspase-specific activities. The cleaved caspase-3 and PARP expressions were down-regulated in

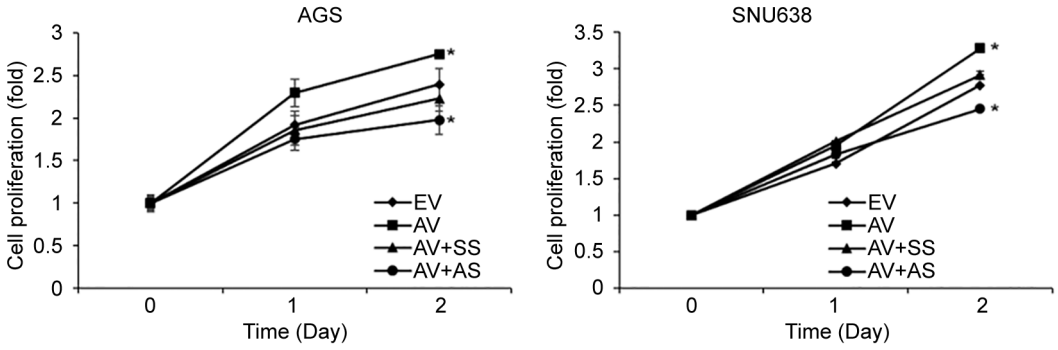

Figure 3. *Impact of A disintegrin and metalloprotease 12 (ADAM12) on the proliferation of gastric cancer (GC) cells. ADAM12 siRNA (AS)-transfected AGS and SNU638 cells significantly decreased the number of proliferating cells compared to that in scramble siRNA (SS)-transfected cells at 0, 1, and 2 days (mean±SD, n=3; *p<0.05). EV, Empty-pcDNA6-myc vector; AV, ADAM12-pcDNA6-myc construct; SD, standard deviation.*

AGS and SNU638 cells after overexpression of *ADAM12* and up-regulated after knockdown of *ADAM12* (Figure 4B).

Impact of ADAM12 on the control of epithelial to mesenchymal transition (EMT) in GC cells. To investigate phenotypic changes induced by the EMT of GC cells, well-known EMT-associated target genes, such as E-cadherin, Snail, and Vimentin were compared regarding overexpression and knockdown of *ADAM12* in AGS and SNU638 cells. The expression of Snail and Vimentin increased and that of E-cadherin decreased after *ADAM12* overexpression. These were reversed after *ADAM12* knockdown (Figure 5). These results indicate that *ADAM12* expression induces molecular and cellular alterations consistent with EMT.

Impact of ADAM12 on oncogenic signaling pathways in GC cells. To examine whether ADAM12 activates intracellular signaling pathways in human colorectal cancer cells, we determined the phosphorylation levels of Akt and GSK-3β signaling proteins using western blotting. We found that the phosphorylation Akt and GSK-3β increased by *ADAM12* overexpression in AGS and SNU638 cells. In contrast, the phosphorylation of Akt and GSK-3β decreased by *ADAM12* knockdown (Figure 6).

Expression of ADAM12 in GC tissues. To confirm the results of GC cell line studies, we evaluated the expression of *ADAM12* at mRNA and protein levels by qRT-PCR and immunohistochemistry in GC tissues and paired normal gastric mucosa. We used the normal gastric mucosa adjacent to the tumor tissue of the GC patient as a paired normal gastric mucosa. In endoscopic biopsy specimens, we confirmed the up-regulation of *ADAM12* expression in GC tissues compared to paired normal mucosa at the mRNA level (p=0.004, Figure 7A). In the paraffin tissue sections, immunostaining of ADAM12 protein was not or was weakly stained in the normal gastric mucosa. The immunostaining of ADAM12 protein was predominantly identified in the cytoplasm of cancer cells and was not detectable in the tumor stroma (Figure 7B).

Association of ADAM12 with the clinicopathological variables of GC. To study the prognostic role of ADAM12 in the progression of GC, we investigated *ADAM12* expression in formalin-fixed paraffin-embedded tissue sections obtained from 212 patients with GC and correlated the results with clinicopathological data of these patients. We analyzed the survival rates of patients with GC and the association between *ADAM12* expression and clinicopathological variables of these patients. We observed that *ADAM12* expression was significantly associated with age, tumor size, cancer stage, depth of invasion, and lymph node metastasis (p=0.001, p<0.001, p<0.001, p=0.001, and p<0.001, respectively; Table I). Overall survival of patients with ADAM12-positive tumors was significantly lower than that of patients with ADAM12-negative tumors (p<0.001, Figure 8).

Association of ADAM12 expression with tumor cell proliferation and apoptosis in GC. All tumor samples were assessed by the TUNEL assay and immunostaining for Ki-67 to determine the apoptosis and proliferation of tumor cells. The apoptotic index (AI) of the 212 tumor samples ranged from 0.1 to 5.1, with a mean AI of 1.71±1.43. No significant difference was observed between *ADAM12* expression and AI (p=0.290). The Ki-67 labeling index (KI) of the 212 tumor samples ranged from 14.8 to 86.5, with a mean KI of 48.23±17.52. The mean KI value of ADAM12-positive tumors was 52.12±17.16, which was significantly lower than that of ADAM12-negative tumors (p=0.030; Table II).

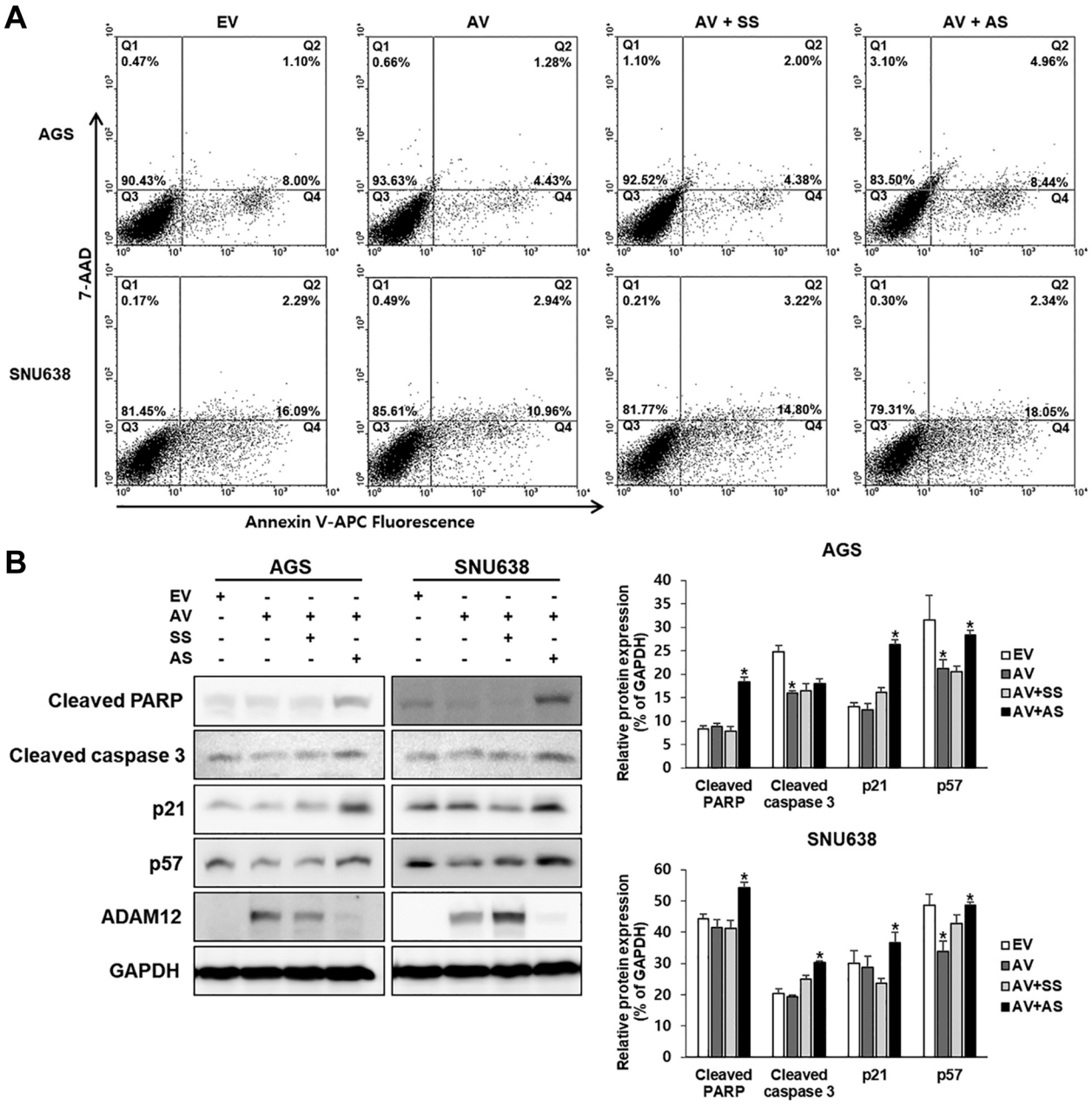

Figure 4. *Impact of A disintegrin and metalloprotease 12 (ADAM12) on apoptosis in gastric cancer (GC) cells. (A) Cell apoptotic rate with ADAM12 expression was analyzed by Annexin V-APC staining and a FACSCalibur flow cytometer. The cell apoptotic rate was significantly decreased in AGS and SNU638 cells after overexpression of ADAM12. Additionally, the cell apoptotic rate induced by the transfection of ADAM12 siRNA (AS) significantly increased compared to that induced by transfection of scramble siRNA (SS) in AGS and SNU638 cells. (B) Impact of apoptotic proteins by ADAM12 expression was demonstrated by western blotting in GC cells. Expression of cleaved caspase3 and poly (ADP-ribose) polymerase (PARP) increased upon ADAM12 knockdown. The expression of target proteins was estimated by three independent experiments using western blotting. Thereafter, images were densitometrically analyzed using the Multi Gauge v3.0 software for quantification of protein expression. EV, Empty-pcDNA6-myc vector; AV, ADAM12-pcDNA6-myc construct.*

Discussion

ADAMs have been implicated in cell adhesion, signaling, migration, and proteolysis (8, 32), and there is growing evidence that specific ADAMs play a crucial role in prognosis or as a predictive biomarker in a variety of cancers (33-37). Expression of *ADAM12* has been found to be increased and correlated with tumor stage and prognosis in colon, breast, lung, bladder, prostate, and liver cancers (22-27). In GC, *ADAM8, ADAM9, ADAM10, ADAM12, ADAM17, ADAM28,*

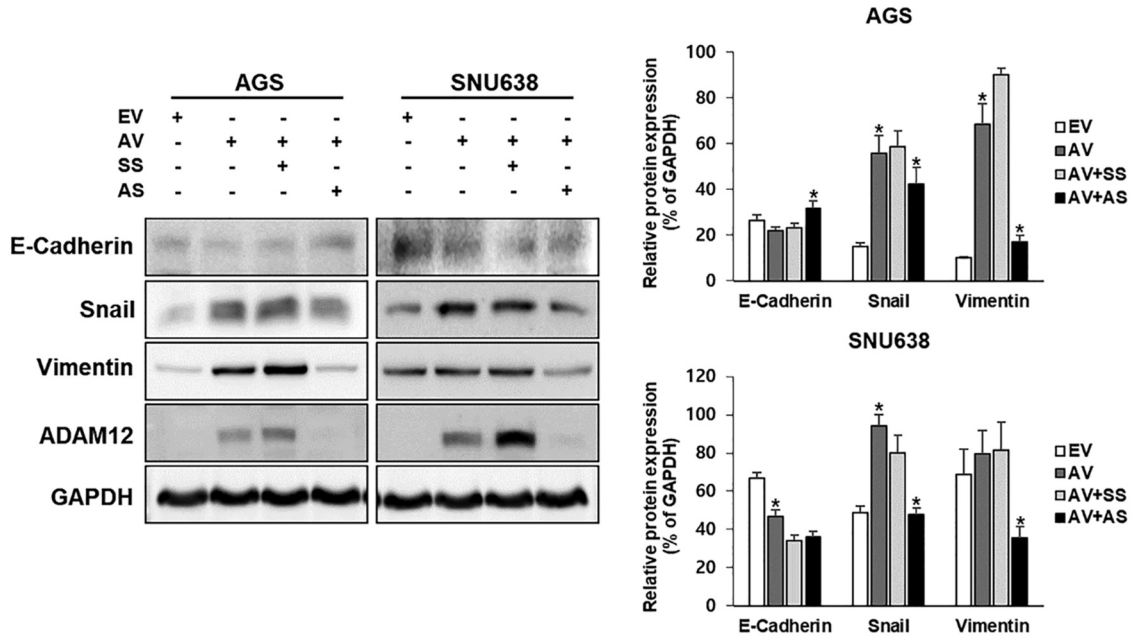

Figure 5. *Impact of A disintegrin and metalloprotease 12 (ADAM12) on the control of epithelial to mesenchymal transition (EMT) in gastric cancer (GC) cells. To investigate phenotypic changes induced by EMT of GC cells, the well-known EMT-associated target genes, such as E-cadherin, Snail, and Vimentin were compared upon and knockdown of ADAM12 in AGS and SNU638 cells. The expression of Snail and vimentin increased and that of E-cadherin decreased after ADAM12 overexpression. These were reversed after ADAM12 knockdown. The expression of target proteins was estimated by three independent experiments using western blotting. Thereafter, images were densitometrically analyzed using the Multi Gauge v3.0 software for quantification of protein expression. EV, Empty-pcDNA6-myc vector; AV, ADAM12-pcDNA6-myc construct; SS, scramble siRNA; AS, ADAM12 siRNA.*

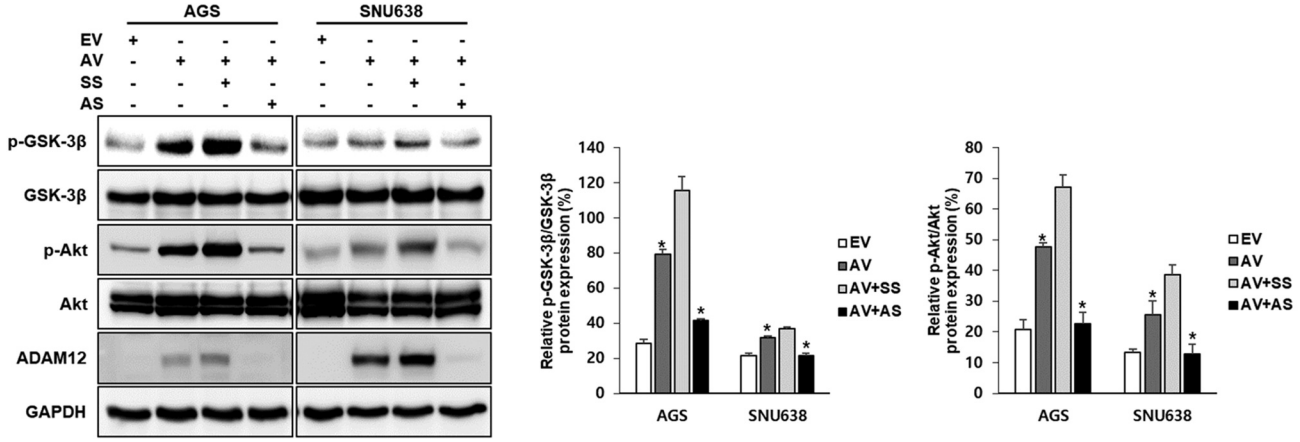

Figure 6. *Impact of A disintegrin and metalloprotease 12 (ADAM12) on oncogenic signaling pathways in gastric cancer (GC) cells. Western blotting showed that the phosphorylation of Akt and glycogen synthase kinase 3β (GSK-3β) increased by ADAM12 overexpression in AGS and SNU638 cells. In contrast, phosphorylation of Akt and GSK-3β decreased by ADAM12 knockdown. The expression of target proteins was estimated by three independent experiments using western blotting. Thereafter, images were densitometrically analyzed using the Multi Gauge v3.0 software for quantification of protein expression. EV, Empty-pcDNA6-myc vector; AV, ADAM12-pcDNA6-myc construct; SS, scramble siRNA; AS, ADAM12 siRNA.*

and *ADAM29* are up-regulated and involved in tumor formation and progression (14-19). However, the role and molecular mechanisms of *ADAM12* in GC is unclear (28-31). Therefore, we investigated whether *ADAM12* may serve as a novel biomarker or a novel therapeutic target in GC using human GC cell lines and GC tissues.

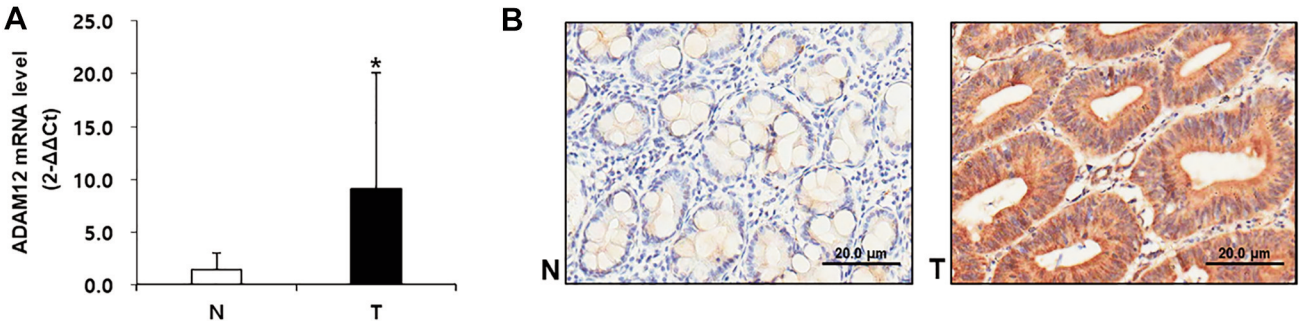

Figure 7. *Expression of A disintegrin and metalloprotease 12 (ADAM12) in gastric cancer (GC) tissues by quantitative real time-polymerase chain reaction (qRT-PCR) and immunohistochemistry. (A) Expression of ADAM12 was up-regulated in cancer tissues compared to paired normal mucosa at the mRNA level in endoscopic biopsy specimens. Each bar represents the mean±SD of 20 cases. *p<0.05 vs. normal gastric mucosa. (B) Immunostaining of the ADAM12 protein was inexistent or weak stained in the normal gastric mucosa (N) (200×). Immunostaining of the ADAM12 protein was predominantly identified in the cytoplasm of tumor cells and was not detectable in the tumor stroma (T) (200×). SD, Standard deviation; N, normal gastric mucosa; T, gastric cancer tissue.*

Table I. *Association of A disintegrin and metalloprotease 12 (ADAM12) with the clinicopathological variables of gastric cancers.*

Variables	Total (n=212)	*ADAM12* Negative (n=116)	*ADAM12* Positive (n=96)	*p*-Value
Age (years)				0.001
<58.47	83	57	26	
>58.47	129	59	70	
Sex				0.792
Male	146	79	67	
Female	66	37	29	
Tumor size (cm^3)				<0.001
<4.27	138	89	49	
>4.27	74	27	47	
Stage				<0.001
I/II	133	87	46	
III/IV	79	29	50	
Histologic type				0.359
Well-differentiated	60	31	29	
Moderately differentiated	56	33	23	
Poorly differentiated	89	50	39	
Mucinous/signet	7	2	5	
Depth of invasion (T)				0.001
T1/T2	116	76	40	
T3/T4	96	40	56	
Lymph node metastasis (N)				<0.001
N0	121	80	40	
N1-3	91	35	56	

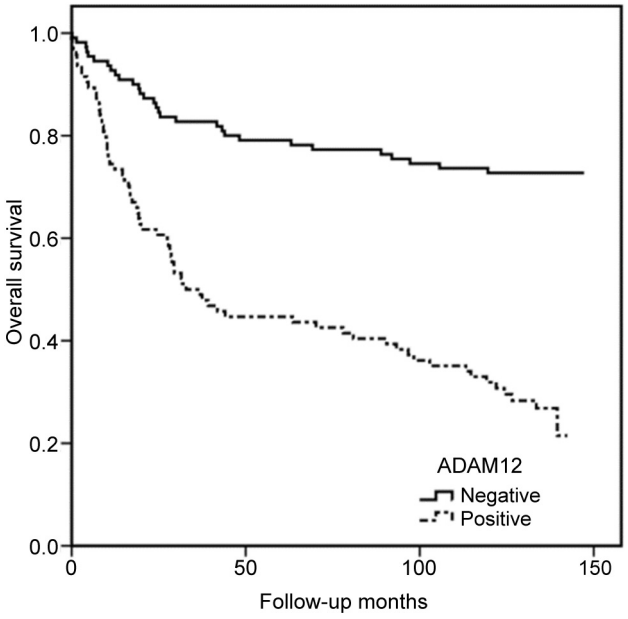

Figure 8. *Kaplan-Meier survival curve correlating overall survival with positive expression (-dotted line) and negative expression (solid line) of A disintegrin and metalloprotease 12 (ADAM12).*

In cancer, the loss of tissue homeostasis and restructuring of the tumor microenvironment are critical discriminants of increased proliferation and resistance toward apoptosis and cell-cycle regulation (38-40). Previous studies reported that up-regulation of *ADAM12* promoted proliferation and inhibited apoptosis of various cancer cells (22-27). In the present study, overexpression of *ADAM12* enhanced proliferation and inhibited the apoptosis *via* down-regulation of caspase 3 and PARP activities, and induced cell-cycle arrest *via* down-regulation of CDK inhibitors p21 and p57 in GC cells. These effects were reversed after the knockdown of *ADAM12*. These results suggest that ADAM12 contributes to alteration of tumor cell survival in GC.

Increased tumor cell migration and invasion are crucial in cancer invasion and metastasis (38). We observed that

Table II. *Association of A disintegrin and metalloprotease 12 (ADAM12) expression with tumor cell proliferation and apoptosis in gastric cancers.*

Indices (mean±SD)	Total (n=212)	*ADAM12* expression		*p*-Value
		Negative (n=116)	Positive (n=96)	
KI	48.23±17.52	36.55±13.61	52.12±17.16	0.030
AI	1.71±1.43	1.24±1.62	1.87±1.36	0.290

KI, Ki-67 labeling index; AI, apoptotic index; SD, standard deviation.

ADAM12 overexpression enhanced tumor cell invasion and migration in GC cells. In contrast, *ADAM12* knockdown suppressed tumor cell invasion and migration. These results indicate that *ADAM12* may have an oncogenic role in GC progression and metastasis.

EMT is a key process involved in cancer development and progression and is characterized by the down-regulation of epithelial markers, such as E-cadherin, and up-regulation of mesenchymal markers, such as Snail and Vimentin (41). In a previous study, *ADAM12* induced EMT, a key process of cancer progression in breast cancer (42) and pituitary adenoma (43). Thus, we evaluated the expression of EMT-associated target genes by western blotting in GC cells. The expression of E-cadherin decreased and that of Snail and Vimentin increased by *ADAM12* overexpression. These were reversed by *ADAM12* knockdown. These results indicate the positive relationship between *ADAM12* expression and induction of EMT in GC cells.

To explore the potential mechanisms involved in these effects of *ADAM12*, we studied the effect of *ADAM12* on the stimulation of multiple intracellular signaling pathways, leading to inhibition of apoptosis and cell cycle progression. PI3K/Akt and GSK-3β signaling pathways play a pivotal role in tumorigenesis, enhancing cell proliferation, angiogenesis, inhibition of apoptosis, and EMT in various human cancers (44-46). *ADAM12* regulates colorectal cancer cell invasion through the PI3K/Akt signaling pathway (22). Additionally, *ADAM12* promotes cell migration, invasion, and proliferation in pituitary adenomas *via* the EGFR/ERK signaling pathway (43). In the present study, the phosphorylation of Akt and GSK-3β increased by *ADAM12* overexpression in GC cells. In contrast, the phosphorylation of Akt and GSK-3β decreased by *ADAM12* knockdown. The results suggest that *ADAM12* may regulate human GC cell behavior through activation of PI3K/Akt and GSK-3β signaling pathway.

In the present study, using GC tissues, *ADAM12* expression in GC tissues was up-regulated compared to paired normal mucosa at mRNA and protein levels in endoscopic biopsy and surgical specimens. Overexpression of *ADAM12* was significantly associated with age, tumor size, cancer stage, depth of invasion, lymph node metastasis, and poor survival in patients with GC. These results, including those of the present study, suggest that *ADAM12* expression may help in predicting a poor clinical outcome of human GC.

Finally, we evaluated the correlation between *ADAM12* expression and tumor cell proliferation and apoptosis in GC tissues to confirm the results of GC cell line studies. The Ki-67 protein is a well-known marker for cell proliferation and can be used in immunohistochemistry (47). Previous studies have shown that KI could be a potential predictive biomarker for clinical outcomes in patients with GC (48, 49). In the present study, the mean KI value of ADAM12-positive tumors was significantly higher than that of ADAM12-negative tumors. However, there was no significant difference between *ADAM12* expression and AI. Therefore, these results confirm the *in vitro* enhancement of the cell proliferation-inducing potential of *ADAM12 in vivo*.

Conclusion

ADAM12 promotes tumor development and progression by increasing mobility, enhancing proliferation, and inhibiting apoptosis in GC cells. Moreover, up-regulation of *ADAM12* is associated with adverse clinicopathological features and poor survival. It may be used as a molecular marker for the prediction of the clinical outcomes of patients with GC.

Conflicts of Interest

The Authors declare that they have no conflicts of interest.

Authors' Contributions

Conceptualization: Min-Woo Chung and Young-Eun Joo. Performed most of the experiments: Young-Lan Park, Sun-Young Park. Designed the experiments and drafted the article: Min-Woo Chung and Young-Eun Joo. Supervision: Young-Eun Joo. Approval of final article: all Authors.

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