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Identification of S100A8 and S100A9 as negative regulators for lymph node metastasis of gastric adenocarcinoma

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Summary. With increasing therapeutic use of minimally invasive therapy for treatment of early gastric cancer, prediction of lymph node metastasis is important. In search of tissue biomarkers for prediction of lymph node metastasis of gastric adenocarcinoma, we analyzed gastric adenocarcinoma tissue using proteomic methods. We have done 2D-PAGE and MALDI-TOF MS analysis in matched normal and gastric cancer tissues. We also evaluated the clinicopathological significance of expression of S100A8 and S100A9 in gastric adenocarcinoma using a tissue microarray of 218 gastric adenocarcinoma specimens. Cell invasion and migration assay were performed to confirm functional role of S100A8 and S100A9 using small hairpin RNA lentivirus. We identified 8 up-regulated and 5 down-regulated proteins in gastric cancer tissues compared to matched normal mucosa. Of these, expression of S100A8 and S100A9 occurred mainly in stromal cells and inflammatory cells between tumor cells. Correlation was observed between small lesion size, decreased depth of invasion, a tendency to absence of lymphovascular tumor emboli, a decrease in perineural invasion and lymph node metastasis, and expression of stromal S100A8. In addition, increased expression of stromal S100A9 in gastric adenocarcinoma was associated with small lesion size and a decrease in lymph node metastasis. Functional analysis confirmed that down-regulation of S100A8 and S100A9 by small hairpin RNA lentivirus induced an increase of migration and invasion in gastric cancer cell lines. Taken together, these findings suggest that S100A8 and S100A9 are negative regulators of lymph node metastasis of gastric adenocarcinoma and can be used as biomarkers for prediction of lymph node metastasis in gastric adenocarcinoma.

Key words: Stomach, Adenocarcinoma, S100A8, S100A9, Lymph node metastasis

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Introduction

With improvements in endoscopic techniques, novel therapeutic modalities, such as endoscopic mucosal resection (EMR) and endoscopic submucosal dissection (ESD), have been developed for the treatment of gastric cancer. EMR/ESD procedures are currently widely accepted treatment modalities for early gastric cancer, mostly in Asia, including Korea and Japan (Gotoda et al., 2006; Gotoda, 2007). For gastric adenocarcinomas, widely accepted indications for EMR/ESD are intestinal (differentiated) type cancer, limited to the mucosa, measuring up to 2 cm in diameter and without lymphovascular tumor emboli (Gotoda et al., 2006; Gotoda, 2007). These indications are based on large Japanese datasets indicating an almost absent risk of lymph node metastasis in lesions such as the one described above (Gotoda et al., 2000). Several radiological and molecular methods have been used to predict progression of EGCs, i.e., submucosal invasion and lymph node metastasis. Endoscopic ultrasonography is used widely in staging of EGC, and, thus, detection of submucosal invasion and lymph node metastasis (Shimoyama et al., 2004). Various clinicopathological

features (size, histology), but also protein expression, have been reported as biological markers of submucosal invasion and lymph node metastasis, including mucin phenotypes and beta-catenin expression (Gotoda et al., 2000; Miyazawa et al., 2000; Kabashima et al., 2002; Otsuji et al., 2007). However, controversies exist among investigators regarding the validity of these biological markers.

With this information in hand, we attempted to find new candidate biomarkers associated with lymph node metastasis in gastric cancer tissue using proteomic methods, two-dimensional electrophoresis (2-DE) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Among the proteins that were found to be elevated in gastric cancer tissue, S100A8 and S100A9 were selected for further validation of their roles in lymph node metastasis in gastric adenocarcinoma.

Materials and methods

Proteomic analysis of gastric cancer tissue

A total of 4 tumor tissues and 4 matched adjacent mucosal tissues were collected from 4 patients with gastric adenocarcinoma (all intestinal type advanced gastric cancer tissue). We selected fresh gastric cancer tissues containing at least 60 % tumor cells without mucin, or normal tissue contamination by mirror image analysis.

These fresh tissues were obtained from National Biobank of Korea, PNUH, and approval was obtained from the Institutional Review Board. 2-D-PAGE was carried out roughly as described. Aliquots in sample buffer (7 M urea, 2 M thiourea, 4.5% CHAPS, 100 mM DTE, 40 mMTris, pH 8.8) were applied to immobilized pH 3–10 nonlinear gradient strips (Amersham Biosciences, Uppsala, Sweden). IEF was performed at 80,000 Vh. The second dimension was analyzed on 9-16% linear gradient polyacrylamide gels (18 cm 6 20 cm 6 1.5 mm) at constant 40 mA per *gel* for approximately 5h. After protein fixation in 40% methanol and 5% phosphoric acid for 1h, gels were stained with CBB G-250 for 12 h. Gels were de-stained with H_2O, scanned in a Bio-Rad (Richmond, CA) GS710 densitometer, and converted into electronic files, which were then analyzed using the Image Master Platinum 5.0 image analysis program (Amersham Biosciences).

For MALDI-TOF MS analysis, peptides were concentrated by a POROS R2, Oligo R3 column (Applied Biosystems, Fostercity, CA, USA). After washing the column with 70% acetonitrile, 100% acetonitrile, and then 50 mM ammonium bicarbonate, samples were applied to the R2, R3 column and eluted with cyano-4-hydroxycinamic acid (CHCA) (Sigma, St. Louis, MO) dissolved in 70% acetonitrile and 2% formic acid onto the MALDI plate (Opti-TOF™ 384-well Insert, Applied Biosystems). MALDI-TOF MS was performed on a 4800 MALDI-TOF/TOF™ Analyzer (Applied Biosystems) equipped with a 355-nm Nd:YAG laser. The pressure in the TOF analyzer was approximately 7.6e-07 Torr. Mass spectra were obtained in the reflectron mode with an accelerating voltage of 20 kV, sum from 500 laser pulses and calibrated using the 4700 calibration mixture (Applied Biosystems). Data Explorer 4.4 (PerSeptiveBiosystems) was used for data acquisition and extraction of the monoisotopic masses.

Gastric cancer cell lines

Human gastric cancer cell line AGS cells were obtained from KCLB (Korean Cell Line Bank) and cultured in RPMI1640 medium with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. All cells were maintained at 37°C in 5% CO_2.

Real time RT-PCR analysis of S100A8 and S100A9 in gastric cancer tissue and gastric cancer cell lines

RNA was extracted from gastric cancer tissues and cells using the RNeasy plus kit (Qiagen, Hilden, Germen) and TRI reagent (Sigma, St. Louis, MO, USA), respectively, according to the manufacturer's instructions. cDNA was synthesized from 2 μg total RNA using Patimum PCR SuperMix (Invitrogen, CA, USA) in a final volume of 20 μl. For each gene, the sequences of forward and reverse primers are as follows: S100A8 (forward, 5'-ATGCCGTCTACAGGGATGAC; reverse, 5'-ACGCCCATCTTTATC ACCAG); and S100A9 (forward, 5'-TCATCAACACCTTCCACCAA; reverse, 5' GTTAGCCTCGCCATCAGCAT), ß-actin (QT00095431, QIAGEN) and gapdh (forward, CATCTTCCAGGAGCGA; reverse, ACCACCAA CTGCTTAG). PCR cycling conditions were 10 minutes at 95°C, followed by 35 cycles of 30 seconds at 95°C, 30 seconds at 53°C (for gapdh) and 56°C (for S100A8, S100A9 and ß-actin), and 30 seconds at 72°C. The threshold cycle (CT) is defined as the fractional cycle number at which the fluorescence passes a fixed threshold above baseline. Relative S100A8 and S100A9 gene expression quantification for gastric adeno-carcinoma tissue and matched surrounding normal tissue of each sample was calculated using the average S100A8 and S100A9 CT value for each triplicate sample minus the average triplicate CT value for ß-actin, and differences between gastric adenocarcinoma tissue and matched surrounding normal tissue were calculated using the formula 2- ($\Delta^{CT\ tumor-}\ \Delta^{CT\ normal}$) and expressed as a fold change in expression according to the comparative threshold cycle method (2-Δ^{CT}) (Livak and Schmittgen, 2001). RT-PCR products were then subjected to 1% agarose gel electrophoresis and stained with ethidium bromide.

ShRNA lentivirus-mediated silencing and overexpression of S100A8 and S100A9 in gastric cancer cell lines

Lentiviral-based RNA knockdown or overexpression

approaches were used for silencing or overexpression of S100A8 and S100A9 in gastric cancer cell lines. The non-target shRNA control vector, pLKO-non target, pLenti-human S100A8 shRNA vectors and pLenti-human S100A9 shRNA vectors, lentiviral vector PLKO S100A8, S100A9 and empty PLKO vector were purchased from Sigma-Aldrich (St. Louis, MO, USA). Lentivirus stocks were produced using Virapower™ lentivirus packaging mix and the 293FT cell line according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). AGS cell lines which were grown to 50% confluence were incubated for 24 hr 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 S100A8, S100A9 was examined by RT-PCR.

Cell invasion assay

The chemotactic cell invasion assay was performed using 24-well Transwell units with an 8-μm pore size polycarbonate filter (Corning Incorporated, Costar), as described previously. The upper side of the polycarbonate filter was coated with Matrigel (5 mg/ml in cold medium) to form a continuous thin layer. Prior to addition of the cell suspension, the dried layer of Matrigel matrix was rehydrated with serum-free RPMI for 3 hr at 37°C. Each lower compartment of the Transwell contained 600 μl RPMI supplemented with 5% FBS as a chemo-attractant. Serum-starved gastric cancer cells ($1x10^5$) were suspended in 0.1 ml RPMI and added to the upper compartment of the Transwell unit and incubated for 18 hr at 37°C in a humidified atmosphere containing 5% CO_2. Cells remaining in the upper compartment were completely removed with gentle swabbing. The number of cells that had crossed the filter to the lower compartment was determined using staining with Hoechst 33258.

Cell migration assay

Migration of gastric cancer cells was assayed using a Boyden chamber apparatus, as previously described. Briefly, gastric cancer cells were harvested with 0.05% trypsin containing 0.02% ethylenediaminetetraacetic acid, washed once, and suspended in RPMI at a concentration of $1x10^4$ cells/ml. An 8-μm poresize polycarbonate membrane filter contained in a disposable 96-well chemotaxis chamber (Neuro Probe, Gaithersburg, MD) was pre-coated for 4 h with 5 mg/ml fibronectin at room temperature; 50 μl of gastric cancer cell suspension was loaded into the upper chamber, and 5% FBS was loaded into the lower or upper chamber. After incubation of cells for 18 hr at 37°C, the filter was disassembled and the upper surface was scraped free of cells by wiping with a cotton swab. The number of cells that had migrated to the lower surface of the filter was determined by counting the cells in 4 places at x100 magnification after staining with Hoechst 33258.

Clinicopathological analysis and tissue microarray in gastric adenocarcinoma

A total of 218 gastric cancer patients who underwent gastrectomy with lymph node dissection at Pusan National University Hospital between 2007 and 2008 form the basis of this study. The group was composed of 153 male and 65 female patients with a mean age of 59.0 years (42-75 years). Formalin-fixed and paraffin embedded specimens were obtained in all cases. We assessed the following clinicopathological factors; sex, age, site, gross type, presence of ulceration, tumor size, depth of invasion, histological classification (intestinal and diffuse), and lymphovascular invasion based on Korean guidelines of stomach adenocarcinoma (Kim et al., 2005). A semi-automated tissue arrayer (Beecher Instruments) was used for construction of a tissue microarray. We made two tissue cores, 0.6 mm in diameter, which were taken from representative tumor blocks in each case of gastric adenocarcinoma. No selection was made between more invasive front areas and central tumor areas.

Immunohistochemistry and interpretation of results

Sections of tissue microarray were de-waxed and rehydrated according to standard procedure, and washed with PBS. The sections were first heated in a microwave oven at 600W for 2x5 minutes in 0.01M citrate buffer, pH 6.0. The sections were then immersed in 3% H_20_2 to quench endogenous peroxidase activity, and non specific binding was blocked in 5% normal goat serum (0.1% BSA in PBS). Immunohistochemical staining was performed by the avidin-biotin peroxidase complex method with aminoethylcarbazole as a chromogen using the Vetastain ABC elite kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's instructions. Sections were counterstained with Mayer's hematoxylin solution. Immunohistochemical staining was carried out with monoclonal antibodies against S100A8 and S100A9. We performed semiquantitative evaluation of expression of S100A8 and S100A9 staining intensity (absent to faint, moderate and strong) and areas of positive areas in gastric adenocarcinoma. For assessment of the positivity of immunostaining, we defined over 30% of positive stromal areas and moderate and strong intensity.

Statistical analysis

Data were analyzed by Student's t test, Fischer's exact test, or χ^2 test for differences between groups. $p<0.05$ was considered statistically significant. Statistical calculations were performed using SSPS version 10.0 for Windows software (SPSS Inc., Chicago, IL, USA).

Results

Proteomic analysis of gastric cancer tissues

A total of 4 tumor tissues and 4 matched adjacent mucosal tissues were collected from 4 gastric adenocarcinoma patients. Around 800 proteins were detected on each 2DE gel. Statistical analysis of 4 DIGE experiments showed changes of expression of 13 proteins by more than 2-fold within the 95% confidence level (Student's t-test; $p<0.05$) of individual variation (Fig. 1). Among the 13 proteins, 8 were increased and 5 were decreased in their expression. Table 1 summarizes the mass spectrometric results.

Expression and cellular localization of S100A8 and S100A9 in gastric adenocarcinoma

We focused on S100A8 and S100A9 for their roles in gastric carcinogenesis; in particular, increased expression of S100A8 and S100A9 mRNA was observed in gastric cancer tissue, compared with their matched adjacent mucosal tissue (Table 2). The results of immunohistochemistry revealed protein expression of high levels of S100A8 and S100A9 in tumor tissue compared with normal tissue. Localization and expression of S100A8 and S100A9 was also evaluated by immunohistochemistry. S100A8 and S100A9 were mostly expressed in stromal cells and inflammatory cells between tumor cells. Some positive cells were observed in tumor cells, probably due to diffusion. Adjacent gastric non-cancerous mucosal tissue showed only some scattered S100A8 and S100A9-positive cells in the lamina propria (Fig. 2).

Relationship between expression of S100A8 and S100A9 and clinicopathological characteristics of gastric adenocarcinomas

We evaluated the clinicopathological significance of expression of S100A8 and S100A9 in gastric adenocarcinoma. We performed semiquantitative evaluation of expression of staining intensity and areas of positive stromal cells in tissue microarray specimens. The cutoff for positivity was set at 30%. Among 211 gastric cancer specimens, 88.5% (193/218) and 88.5% (193/218) were positive for S100A8 and S100A9, respectively. In regard to S100A8 expression in gastric adenocarcinoma, correlation was observed between small lesion size ($p=0.032$), decreased depth of invasion ($p=0.015$), a tendency of absence of lymphovascular tumor emboli ($p=0.090$), a decrease in perineural invasion ($p=0.003$) and lymph node metastasis (0.018), and expression of stromal S100A8 of gastric adenocarcinoma (Table 3). In addition, increased expression of stromal S100A9 in gastric adenocarcinoma was associated with small size of the lesion ($p=0.091$) and a decrease in lymph node metastasis ($p=0.018$) (Table 4). In regard to lymph node metastasis, Table 3 and Table 4 show that the N2, N3 groups (12/61, 19.7%) showed a statistically significant decrease of S100A8, S100A9 expression compared to the N0,N1 groups (13/157, 8.3%) ($p=0.018$).

Regulation of migration and invasion of gastric cancer cells by S100A8 and S100A9

Lentiviral-based RNA knockdown or overexpression approaches were used for determination of the roles of S100A8 and S100A9 in invasion and migration of gastric cancer cells (GCs). AGS was infected with lentivirus expressing either non-target, S100A8 targeted, or S100A9 targeted shRNAs for silencing. Lentiviral vector PLKO S100A8, S100A9 and empty PLKO vector were used for overexpression of S100A8 and S100A9 in AGS cells. Polyclonal stable cell lines were selected using puromycine. Expression of S100A8 and S100A9 was determined using RT-PCR; we then obtained stable S100A8 knockdown cell lines (shA8#1 and shA8#5) and stable S100A9 knockdown cell lines (shA9#3 and

Table 1. Mass spectrometric identification of proteins exhibiting altered expression in gastric adenocarcinoma.

Spot No.	GenBank Identification No.	Protein Identification	Ratio (-fold)	MW (kDa)	pI	Score	Match Peptide	Sequence Coverage (%)
817	114635315	proteasome 26S non-ATPase subunit 13 isoform 3	2.2	376	5.53	112	15	43
836	34039	keratin 19	3.3	400	5.04	285	29	56
847	51491207	hypothetical protein	-3.9	401	5.47	68	11	29
1208	22219225	Chain A,Ciystal Structure Analysis Of The Hca li Mutant T199p In Complex	-3.9	260	6.87	103	11	57
1290	1042151	anti-herpes simplex virus glycoprotein B Ig heavy chainvariable region	-2.4	124	8.64	70	6	59
1435	56204911	solutecarrier family 9	-9.7	224	5.55	72	8	33
1546	5453541	anterior gradient 2 homolog	-3.0	175	9.03	76	11	48
1683	20150229	Chain A, Crystal Structure Of The Mrp14 (S100A9)	3.6	113	5.71	112	14	85
1710	20150229	Chain A, Crystal Structure Of The Mrp14 (S100A9)	5.0	113	5.71	96	11	82
1797	21614544	S100 calciurn-binding protein A8	5.6	93	6.51	72	8	68
1816	82568903	UBN2 protein	2.0	901	9.72	70	10	15
1850	48425521	Chain B,Tsg101l(Uev) DomainInComplex With Ubiquitin	2.0	76	5.77	83	7	69
1911	41872527	diacylglycerol kinase zeta isoform 3	3.2	895	8.16	66	15	23

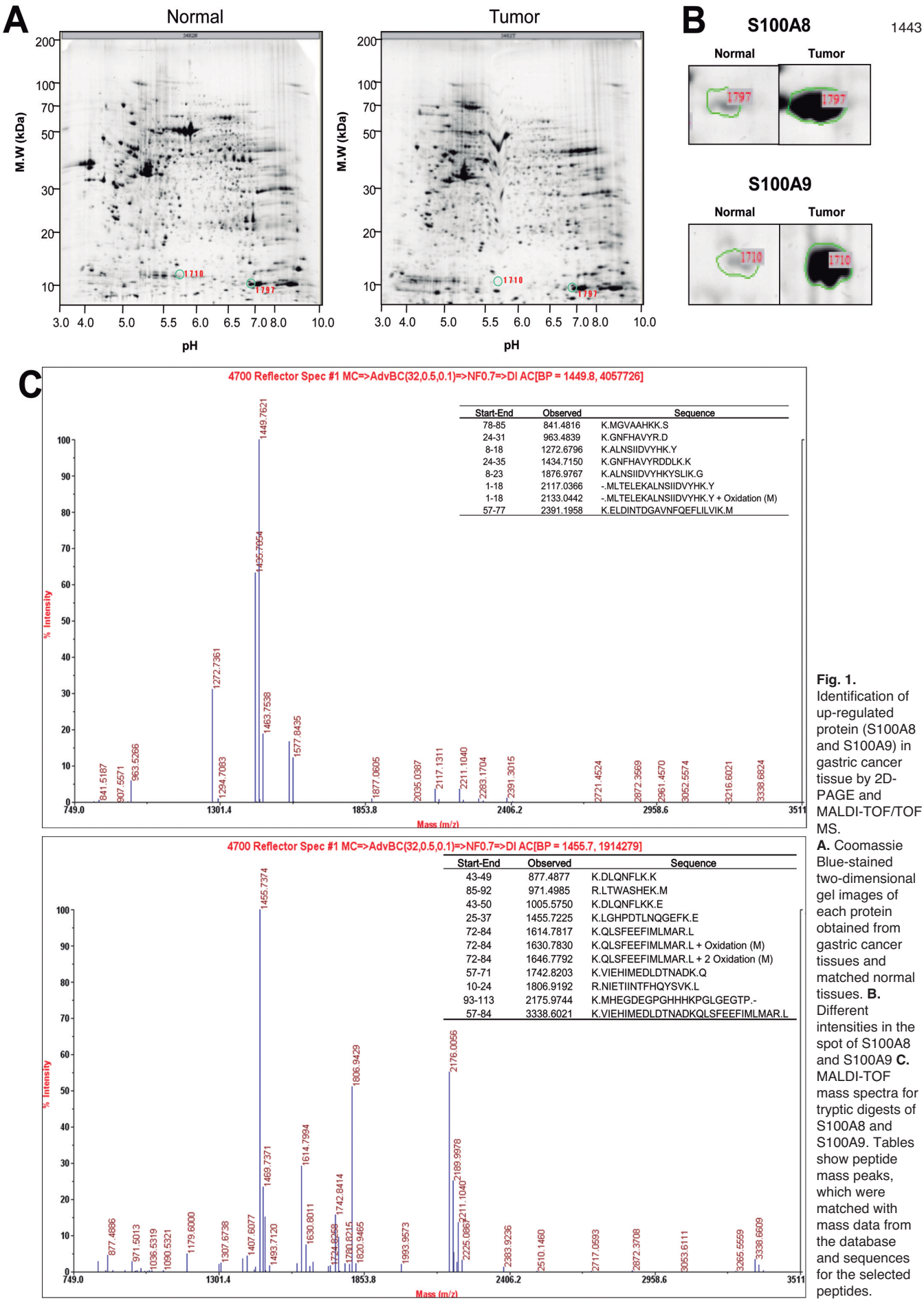

Fig. 1. Identification of up-regulated protein (S100A8 and S100A9) in gastric cancer tissue by 2D-PAGE and MALDI-TOF/TOF MS. **A.** Coomassie Blue-stained two-dimensional gel images of each protein obtained from gastric cancer tissues and matched normal tissues. **B.** Different intensities in the spot of S100A8 and S100A9 **C.** MALDI-TOF mass spectra for tryptic digests of S100A8 and S100A9. Tables show peptide mass peaks, which were matched with mass data from the database and sequences for the selected peptides.

shA9#4) , stable S100A8 overexpression cell lines (OEA8) and S100A9 overexpression cell lines (OEA9) (Figs. 3, 4).

Table 2. Expression of S100A8 and S100A9 mRNA in gastric adenocarcinoma.

	ΔC_T of Tumor	ΔC_T of Normal	fold change ($2^{-(\Delta CT\ Tumor\ -\Delta CT\ Normal)}$)	Mean ± Standard Deviation
S100A8	6.11	7.89	3.43	2.41±0.54
	4.68	5.36	1.60	
	2.52	3.67	2.20	
S100A9	5.12	7.49	5.17	4.67±1.66
	4.79	5.45	1.58	
	1.11	3.98	7.26	

To determine the roles of S100A8 and S100A9 in invasive capacity of gastric cancer cells, we measured chemotactic invasion of the cells using Transwell systems pre-coated with Matrigel. In regard to measurement effect on migration of gastric cancer cells, we performed a cell migration assay using a Boyden chamber apparatus. Of particular interest, tranfection of shRNA for S100A8 or S100A9 showed increased invasion and migration of AGC cells (Figs. 3,4). In contrast to silencing of S100A8 and S100A9, overexpression of S100A8 or S100A9 revealed a decrease in migration, and invasion (only in S100A8) of AGS cells, as shown in Figs. 3, 4.

Discussion

In this study, we attempted to find new candidate biomarkers in gastric cancer tissue using proteomics

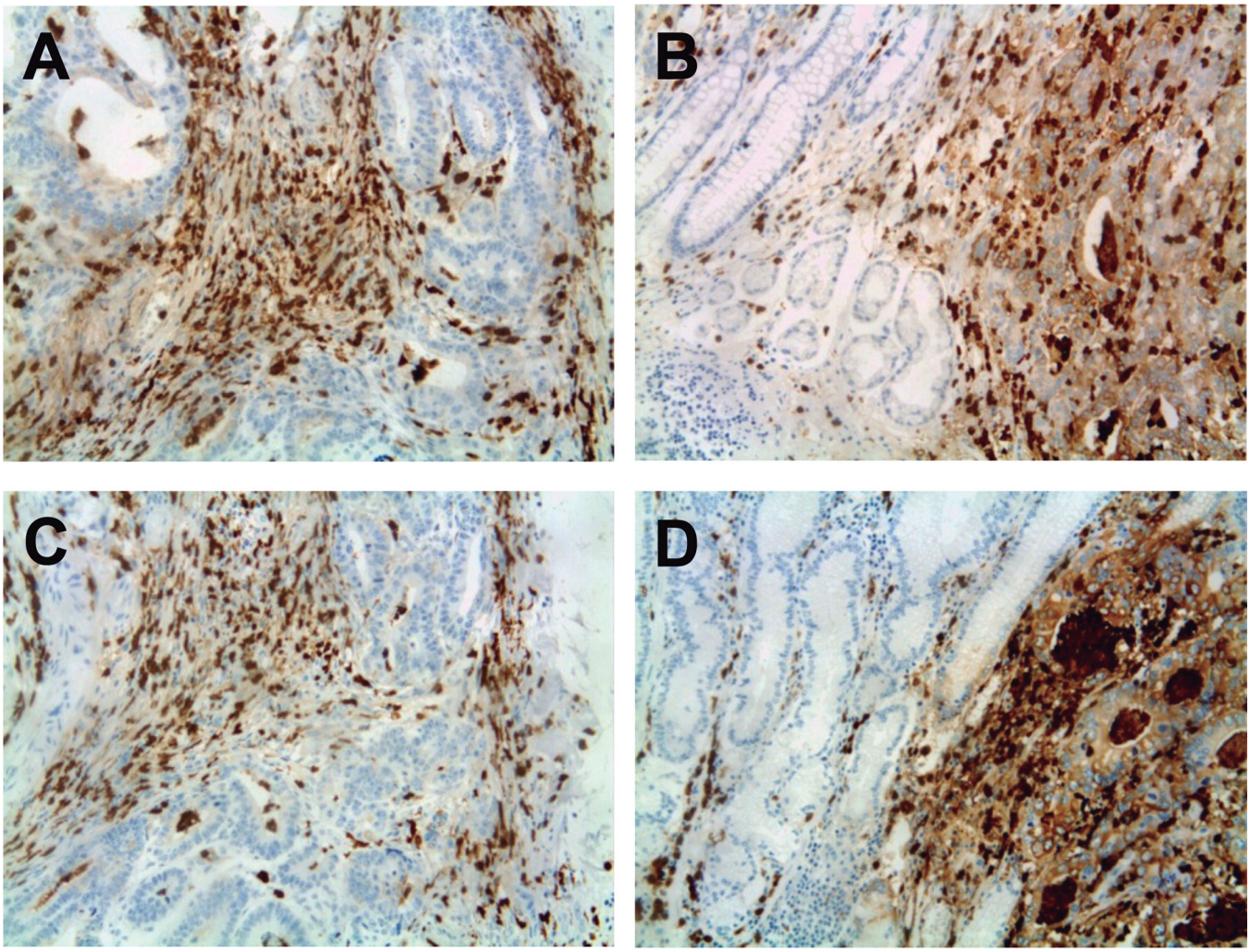

Fig. 2. Immunohistochemical analysis of gastric cancer tissues for S100A8 and S100A9. Localization and expression of S100A8 (**A and B**) and S100A9 (**C and D**) were also evaluated by immunohistochemistry. S100A8 and S100A9 were mostly expressed in stromal cells and inflammatory cells between tumor cells. Some positive cells were seen in tumor cells, probably due to diffusion. A, C, x 100; B, D, x 400

(2D-PAGE and MALDI-TOF/TOF MS), particularly proteins associated with metastasis of gastric adenocarcinoma. By comparing gastric adenocarcinoma tissue with matched normal tissue, we identified 8 up-regulated and 5 down-regulated proteins. Of these, S100A8 and S100A9 were further evaluated. S100A8 and S100A9 were mostly expressed in infiltrating cells in the stroma between tumor cells and showed correlation with decrease of lymph node metastasis in gastric adenocarcinoma. Knock-down of S100A8 and S100A9 by small hairpin RNA lentivirus induced an increase of migration and invasion in gastric cancer cell lines, in agreement with clinicopathological analysis data.

Several gene expression studies and proteomic studies of gastric cancer tissues have been reported (El-Rifai et al., 2002; Que et al., 2004; Li et al., 2008; Jia et al., 2009). A number of proteins with altered expression in gastric cancer tissue were identified in comparison with normal gastric mucosa. Among altered proteins identified in a previous study (El-Rifai et al., 2002), S100A8 and S100A9 were matched with our study and these findings suggested that our 2D-PAGE and MALDI-TOF/TOF MS in gastric cancer tissue was reliable and comparable to previous studies. In addition, our focus was on microenviromental regulation of tumor metastasis, and we selected S100A8 and S100A9 for that reason.

In view of the microenvironmental regulation of metastasis, metastasis processes are influenced by non-malignant cells of the tumor (Joyce and Pollard, 2009). Many of these non-malignant cells are stromal fibroblasts, and a variety of bone marrow-derived cells, including macrophages, myeloid-derived suppressor

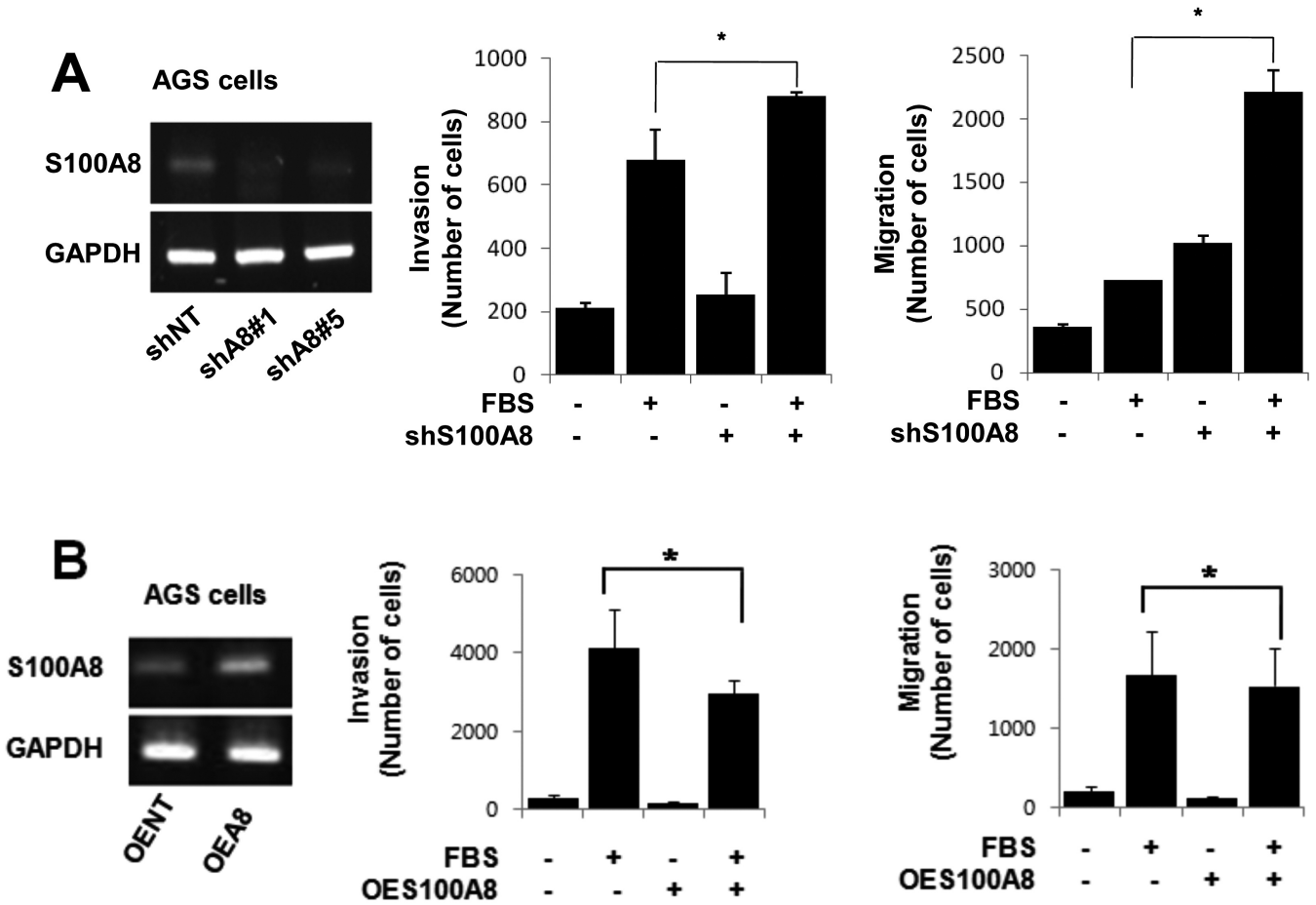

Fig. 3. Role of S100A8 in invasion and migration of GCs. **A.** AGS cells were infected with lentivirus expressing either non-target shRNA (shNT) or one of five S100A8 shRNA (shA8) on day zero, and then harvested on day 7 post-infection. Total RNA isolated from cells was used for RT-PCR analysis for determination of knockdown of S100A8 and two cell lines were generated (shA8#1 and shA8#5). Silencing of S100A8 in AGS cells induced increased migration and invasion. **B.** AGS cells were infected with lentivirus expressing either lentiviral vector PLKO S100A8 or empty PLKO vector (OENT) on day zero, and then harvested on day 7 post-infection. Total RNA isolated from cells was used for RT-PCR analysis for determination of overexpression of S100A8 and one cell line was generated (OEA8). Overexpression of S100A8 in AGS cells induced a decrease in migration and invasion. * indicates $p<0.05$ by Student t-test.

cells, TIE2-expressing monocytes, and mesenchymal stem cells. However, molecular controlling mechanisms of microenvironmental regulation on the metastatic process remain poorly understood. Of these regulating cytokines or factors in the microenvironment, S100A8 and S100A9 are recognized as pro-metastatic cytokines during the metastatic process and members of the S100 multigenic family of non-ubiquitous cytoplasmic calcium-binding proteins of EF-hand type (Gebhardt et al., 2006; Salama et al., 2008; Ghavami et al., 2009). Increased S100A8 and S100A9 has been reported in gastric cancer (El-Rifai et al., 2002), colon cancer (Kim et al., 2009), prostate cancer (Hermani et al., 2005), and breast cancer (Arai et al., 2008). Controversy exists with regard to the roles of S100A8 and S100A9 in carcinogenesis, which is often debated. In general, S100A8 and S100A9 are powerful apoptotic agents produced by immune cells (Ghavami et al., 2008a). S100A8 and S100A9-expressing phagocytes accumulate invasive fronts of malignant tissue and are involved in the immune response against tumors by inducing apoptosis and regression of tumor cells. Contrary to the above roles in carcinogenesis, S100A8 and S100A9 serve as proliferation signals of tumor cells and guide adhesion and invasion of disseminating malignant cells (Ghavami et al., 2008b; Hiratsuka et al., 2006).

In our study, S100A8 and S100A9 were mostly expressed in infiltrating cells in the stroma between tumor cells and showed a correlation with the decrease of lymph node metastasis in gastric adenocarcinoma. In regard to expression of S100A8 and S100A9 in gastric cancer tissue, El-Rifai et al reported over-expression of five calcium-binding proteins (S100A2, S100A7, S100A8, and S100A9) in gastric cancer tissue, compared with normal gastric mucosa, in agreement with our data (El-Rifai et al., 2002). For localization of S100A8 and S100A9, it was reported that S100A8 and S100A9 are expressed in infiltrating cells (myeloid or other inflammatory cells) of tumor stroma in epithelial malignancy (skin, prostate, and colorectal cancers), in agreement with our data (Hermani et al., 2005; Stulik et al., 1999).

Table 3. Relationship between expression of stromal S100A8 expression with clinicopathologic characteristics in gastric adenocarcinoma.

	Case No.	S100A8 expression		p value
		Absent	Present	
Age				
<57	99	10	89	0.563
≥57	119	15	104	
Size				
<4cm	145	12	134	0.032
≥4cm	69	13	59	
Gender				
Male	153	19	134	0.499
Female	65	6	59	
Location				
Upper/Middle	132	20	112	0.034
Lower	86	5	81	
Invasion Depth				
EGC	102	6	96	0.015
AGC	116	19	97	
Gross type				
Elevated/Flat	81	8	73	0.571
Depressed/Excavated	137	17	120	
Histologic Type				
Intestinal	122	14	110	0.819
Diffuse	93	11	83	
Mixed	3	0	3	
Lymphovascular emboli				
Negative	130	11	119	0.090
Positive	88	14	74	
Perineural invasion				
Negative	130	8	122	0.003
Positive	88	17	71	
Lymph node metastasis				
N0,N1	157	13	144	0.018
N2,N3	61	12	49	

Table 4. Relationship between expression of stromal S100A9 expression with clinicopathologic characteristics in gastric adenocarcinoma.

	Case No.	S100A9 expression		p value
		Absent	Present	
Age				
<57	99	11	88	0.880
≥57	119	14	105	
Size				
<4cm	146	13	133	0.091
≥4cm	72	12	60	
Gender				
Male	153	20	133	0.254
Female	65	5	60	
Location				
Upper/Middle	132	20	112	0.034
Lower	86	5	81	
Invasion Depth				
EGC	102	9	93	0.251
AGC	116	16	100	
Gross type				
Elevated/Flat	81	8	73	0.571
Depressed/Excavated	137	17	120	
Histologic Type				
Intestinal	122	16	106	0.604
Diffuse	93	9	84	
Mixed	3	0	3	
Lymphovascular emboli				
Negative	130	13	117	0.408
Positive	88	12	76	
Perineural invasion				
Negative	130	12	118	0.208
Positive	88	13	75	
Lymph node metastasis				
N0,N1	157	13	144	0.018
N2,N3	61	12	49	

For the functional roles in gastric cancer cells, S100A8 and S100A9 were reported to contribute to an increase in invasion, migration, and proteinase expression, in contrast with our data (Yong and Moon, 2007). Our study revealed that knock-down of S100A8 and S100A9 by small hairpin RNA lentivirus induced an increase in migration and invasion in gastric cancer cell lines. Our data showed that S100A8 and S100A9 have roles in the decrease of migration and invasion of gastric cancer cell lines. In addition, these contentions were reinforced by clinicopathological data showing that S100A8 and S100A9 expression were associated with lower frequency of lymph node metastasis in 219 gastric adenocarcinoma cases. In view of the pro-metastatic roles of S100A8 and S100A9 in cancer, primary tumors were reported to secrete VEGF, TGF-beta, and TNF-alpha, which induce S100A8 and S100A9 in myeloid and endothelial cells within lungs prior to metastasis, and that S100A8 and S100A9 act as chemoattractants, which facilitate the homing of tumor cells to premetastatic sites (Hiratsuka et al., 2006, 2008; Rafii and Lyden, 2006). In contrast to these reports, Qin et al reported that S100A8 and S100A9 induced apoptosis and inhibited metastasis in human cervical cancer cell lines, in agreement with our data (Qin et al., 2010). Taken together, the exact and precise mechanisms of S100A8 and S100A9 in metastasis of gastric cancer remain to be elucidated; however, S100A8 and S100A9 might be negative regulators of lymph node metastasis in gastric adenocarcinoma and candidate biomarkers for the prediction of lymph node metastasis in gastric adenocarcinoma.

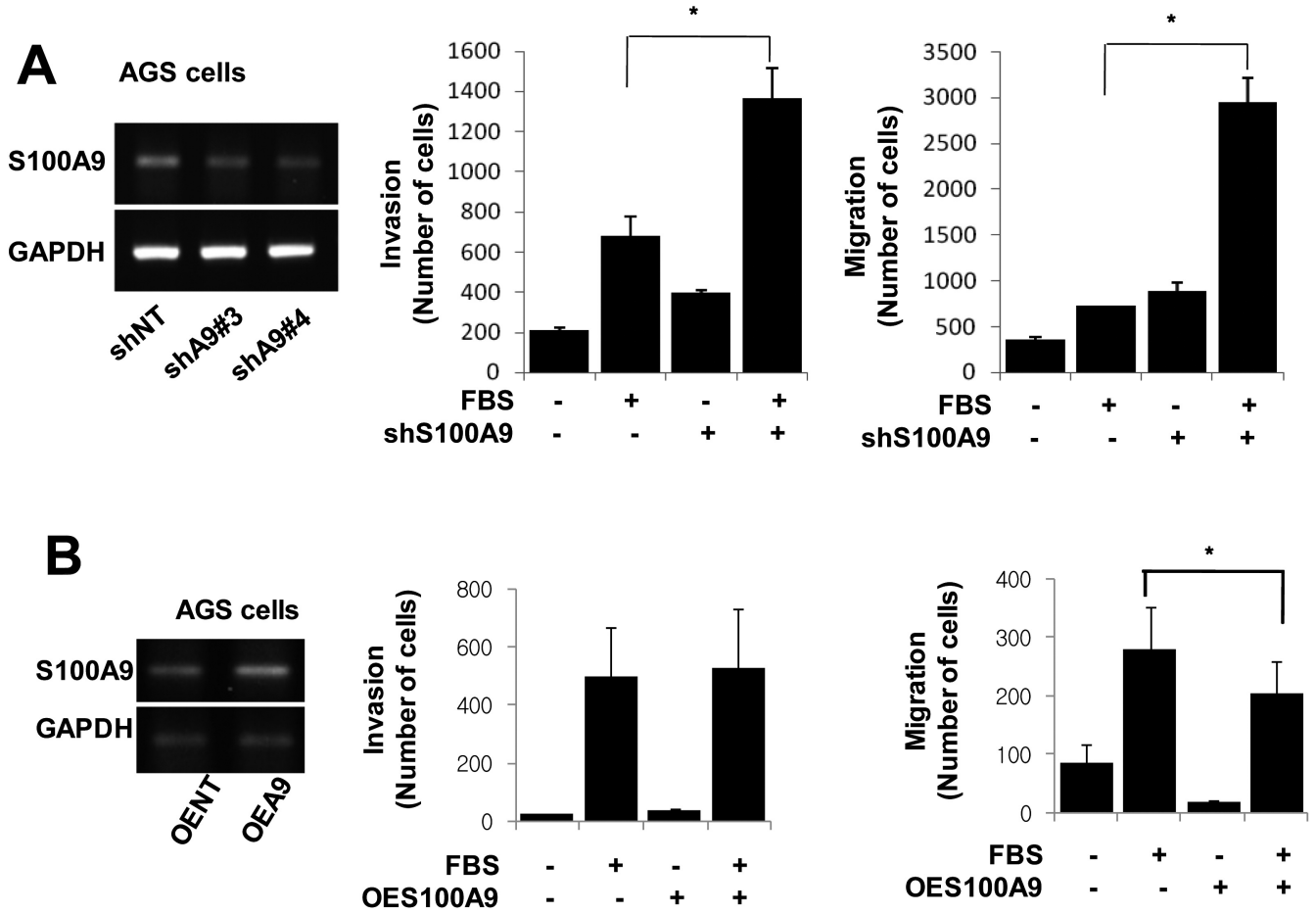

Fig. 4. Role of S100A9 in invasion and migration of GCs. **A.** AGS cells were infected with lentivirus expressing either non-target shRNA (shNT) or one of five S100A9 shRNA (shA9) on day zero, and then harvested on day 7 post-infection. Total RNA isolated from cells was used for RT-PCR analysis for determination of knockdown of S100A9 and two cell lines were generated (shA8#3 and shA8#4). Silencing of S100A9 in AGS cells induced increased migration and invasion. **B.** AGS cells were infected with lentivirus expressing either lentiviral vector PLKO S100A9 or empty PLKO vector (OENT) on day zero, and then harvested on day 7 post-infection. Total RNA isolated from cells was used for RT-PCR analysis for determination of overexpression of S100A9 and one cell line was generated (OEA9). Overexpression of S100A9 in AGS cells induced a decrease in migration. * indicates $p<0.05$ by Student t-test.

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