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# Roles of Calcium-Binding Proteins, S100A8 and S100A9, in Invasive Phenotype of Human Gastric Cancer Cells

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Gastric cancer is one of the most common malignancies and is a frequent cause of cancer-related death in Korea. Cure rate of gastric cancer is quite low because of local invasion and metastasis. S100 proteins are calcium-binding proteins which exert various calcium-mediated cellular functions including cell growth, differentiation, migration and signal transduction. S100A8 and S100A9 are overexpressed in many human tumors and have been shown to be implicated in tumor development or progression. In the present study, we investigated the role of S100A8 and S100A9 in invasive phenotype of a human gastric cancer cell line, SNU484. Expression of S100A8 and S100A9 were detected in SNU484 cells. When the expression of these proteins was suppressed by small-interfering RNA (siRNA) targeting S100A8 or S100A9, the invasive and migratory phenotypes of SNU484 cells were significantly inhibited. The siRNAs for S100A8 and S100A9 inhibited matrix metalloproteinase (MMP)-2 expression in SNU484 cells as evidenced by gelatin zymogram assay, immunoblot analysis and reverse transcription (RT)-PCR. These results demonstrate that S100A8 and S100A9 are required for transcriptional activation of MMP-2 gene in SNU484 cells. Taken together, this study revealed a functional contribution of S100A8 and S100A9 proteins to processes required for malignant progression including invasion, migration and proteinase expression in SNU484 human gastric cancer cells.

**Key words:** S100A8, S100A9, Invasion, MMP-2, Gastric cancer

## INTRODUCTION

S100 calcium-binding proteins are involved in various calcium-mediated cellular functions including cell growth, differentiation, cell migration, cell adhesion, and signal transduction (Li *et al.*, 2004; Arai *et al.*, 2004; Kerkhoff *et al.*, 1999; Li and Bresnick, 2006). Among the S100 proteins, S100A8 (myeloid-related protein-8, MRP8, or calgranulin A) and S100A9 (MRP14 or calgranulin B) have been shown to be implicated in tumor development or progression (Gebhart *et al.*, 2002; Ott *et al.*, 2003; Arai *et al.*, 2004). S100A8 and S100A9 proteins are highly homologous and secreted as a S100A8/A9 heterocomplex (reviewed by Donato *et al.*, 2001). S100A8 and S100A9 are overexpressed in various cancers including gastric cancer (El-Rifai *et al.*, 2002), prostate cancer (Hermani *et al.*, 2005), breast cancer (Cross *et al.*, 2005), lung adenocarcinomas (Arai *et al.*, 2001), pulmonary adenocarcinoma (Arai *et al.*, 2001), and hepatocellular carcinoma (Arai *et al.*, 2000).

Tumor cell invasion and metastasis are complex processes involving extracellular matrix (ECM)-degrading proteinase activity and migration through the ECMs (Fidler, 1990). Mounting evidence suggests a role for members of the matrix metalloproteinase (MMP) family on tumor invasion and metastasis formation, especially, MMP-2 (72 kDa type IV collagenase, gelatinase A) and MMP-9 (92 kDa type IV collagenase, gelatinase B) which degrade type IV collagen, the major structural collagen of the basement membrane (Tryggvason, 1993; Stetler-Stevenson, 1990; Liotta *et al.*, 1991). Numerous studies show a correlation between the levels of MMP-2 and/or MMP-9 and the invasive phenotypes of cancer cells (Ura *et al.*, 1989). Our previous studies suggested the involvement of MMP-2 in H-ras-induced invasive phenotype of MCF10A human breast epithelial cells (Moon *et al.*, 2000; Kim *et al.*, 2003), while H-ras-mediated transformation and invasiveness were associated with enhanced expression of MMP-9 mRNA and protein in rat and human embryonic fibroblasts (Bernhard *et al.*, 1994).

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Gastric cancer is one of the most common malignancies and is a frequent cause of cancer-related death in Korea. Cure rate of gastric cancer is quite low because of local invasion and metastasis (Ji *et al*., 2005). Most patients who are diagnosed with gastric cancer exhibit advanced disease which is often associated with the submucosal invasion of tumor cells (Tsuchiya *et al*., 1995; Yasuda *et al*, 1999). It has been suggested that MMP-2 expression is involved in tumor invasion and metastasis of gastric carcinoma (Nomura *et al*., 1996; Sundlad *et al*., 1998; Monig *et al*., 2001; Takahashi *et al*., 2002). In the present study, we investigated the possible association of S100A8 and S100A9 in the invasive phenotype and MMP-2/-9 expression of a gastric cancer cell line. We provide evidences that S100A8 and S100A9 play crucial roles in invasive phenotype and MMP-2 expression in SNU484 human gastric cancer cells.

## MATERIALS AND METHODS

### Cell lines

Hs683 human glioma cell line (KCLB No. 30138), SK-Hep-1 hepatocellular carcinoma cell line (KCLB No. 30052), MCF7 breast cancer cell line (KCLB No. 30022), and HeLa cervix cancer cell line (KCLB No. 10002) were purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea). T47D breast cancer cell line, LNCaP human prostate cancer cell line and SNU484 gastric cancer cell line were provided by Dr. Y.-J. Surh (Seoul National University, Seoul, Korea), Dr. M.-S. Dong (Korea University, Seoul, Korea), and Dr. H.-D. Um (Korea Institute of Radiological and Medical Science, Seoul, Korea), respectively.

Hs683, SK-Hep-1, MCF7, T47D and HeLa cells were cultured in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum and 100 units/mL penicillin-streptomycin. LNCaP cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum and 100 units/mL penicillin-streptomycin. SNU484 cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum and 50 units/ml gentamicin. Cells were maintained in humidified atmosphere with 95% air and 5% $CO_2$ at 37°C.

### Immunoblot analysis

Equal amounts of protein extracts in SDS-lysis buffer were subjected to 12% SDS-PAGE analysis and electrophoretically transferred to nitrocellulose membrane. Anti-S100A8, anti-S100A9 and anti-MMP-9 antibodies were purchased from Santa Cruz (Santa Cruz, CA). MMP-2 antibody was from R&D system (Minneapolis, U.S.A.). β-actin antibody was from Sigma-Aldrich (St. Lous, MO). Enhanced chemiluminescence (Amersham-Pharmacia, Buckinghamshire, U.K.) system was used for detection. Relative band intensities were determined by quantitation of each band with an Image Analyzer (Vilber Lourmnat, France).

### Synthesis of small-interfering RNA (siRNA) and transfection

The siRNA molecules targeting S100A8 (5'-CCAUCA-UCAACACCUUCCACCAAUA-3') and S100A9 (5'-CCU-UGAACUCUAUCGACGUCUA-3') were purchased from Invitrogen (Carlsbad, CA). Cells were plated in six-well plates at $2\times10^5$ cells/well, grown for 24 h then transfected with each 25 pmole siRNA for 6 h using lipofectamine and OPTI-MEMI reduced serum medium (Invitrogen, Carlsbad, CA). Control cell were treated with Stealth™ RNAi negative control duplex (Invitrogen, Carlsbad, CA).

### Gelatin zymogram assay

Cells were cultured in serum-free medium for 48 h. Gelatinolytic activity of the conditioned media was determined by gelatin zymogram assay as previously described (Moon *et al*., 2000). Areas of gelatinase activity were detected as clear bands against the blue-stained gelatin background.

### *In vitro* invasion assay

*In vitro* invasion assay was performed using 24-well transwell as previously described (Kim *et al*., 2003). The lower side of the filter was coated with type I collagen, and the upper side was coated with Matrigel (Collaborative Research, Lexington, KY). Lower compartment was filled with serum-free media containing 0.1% BSA. Cells were placed in the upper part of the Transwell plate, incubated for 18 h, fixed with methanol and stained with hematoxylin for 10 min followed briefly by eosin. The invasive phenotypes were determined by counting the cells that migrated to the lower side of the filter with microscopy at ×400. 13 fields were counted for each filter and each sample was assayed in triplicate.

### Transwell migration assay

*In vitro* migration assay was performed using a 24 well transwell unit with polycarbonate filters as previously described (Kim *et al*., 2003). Experimental procedures were the same as the *in vitro* invasion assay described above except that the filter was not coated with Matrigel for the migration assay.

### Reverse transcription (RT)-PCR detection

Total RNA was prepared using Trizol reagent (Invitrogen, Carlsbad, CA) according to the Manufacturer's Instruction. Incubated RNA/Oligo dT/dNTP mix was reverse transcribed by RT-Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA). Primers were designed according to the

sequences of MMP-2 and MMP-9 mRNA (GenBank No. NM_004530 and NM_004994, respectively). The sense and antisense primers for MMP-2 were 5'-AATGCC-ATCCCCGATAACC-3' and 5'-AAACTTCACGCTCTTC-AGAC-3', respectively. The sense and antisense primers for S100A9 were 5'-TCTTCCAGTACCGAGAGAAAG-3' and 5'-AGGATGTCATAGGTCACGTAG-3', respectively. For RT-PCR analysis, the following amplification conditions were applied: 94 2 min for 1 cycle, 94 for 30 sec, 50 for 45 sec, 72 for 45 sec for 40 cycles, and 72 for 7 min. Equal volumes of each PCR product were analyzed by agarose gel electrophoresis.

## RESULTS

### Expression of S100A8 and S100A9 in various cancer cell lines

Expression of S100A8 and S100A9 were examined by immunoblot analyses in various human cancer cell lines including Hs683 glioma cell line, SK-Hep-1 hepatocarcinoma cell line, MCF7 breast cancer cell line, T47D breast cancer cell line, SNU484 gastric cancer cell line, LNCaP prostate cancer line and HeLa cervix cancer cell line. Four cell lines out of seven cell lines tested, Hs683, T47D, SNU484 and HeLa cells, expressed both S100A8 (19 kDa) and S100A9 (14 kDa) as shown in Fig. 1. SK-Hep-1 and MCF7 cells expressed only S100A9 but not S100A8. Expression of S100A9 (14 kDa) was observed in all cell lines tested except LNCaP cells.

### Inhibition of S100A8 and S100A9 by siRNA molecules in SNU484 gastric cancer cells

Possible involvement of S100A8 and S100A9 in the malignant tumor progression of gastric cancer was investigated in SNU484 human gastric cancer cell line. To examine the role of S100A8 and S100A9 in SNU484 cells, siRNA molecules targeting S100A8 and S100A9 transcripts were constructed. Selective inhibitory effects of the constructed siRNA molecules on the expression of S100A8 and S100A9 were tested by immunoblot analysis (Fig. 2). When SNU484 cells were transfected with the siRNA for S100A8, protein level of S100A8 was decreased by 47% while that of S100A9 was not significantly affected. The siRNA for S100A9 markedly inhibited the expression of S100A9 (by 85%) without affecting that of S100A8. The data indicate that the siRNA molecules efficiently and selectively inhibited the levels of S100A8 and S100A9 in SNU484 cells.

### Roles of S100A8 and S100A9 in invasive and migratory phenotypes

*In vitro* invasion assay was performed to elucidate the roles of S100A8 and S100A9 in the invasive phenotype of SNU484 cells transfected with either siRNA for S100A8 or siRNA for S100A9. As shown in Fig. 3A, invasiveness of SNU484 cells was significantly inhibited by siRNA for S100A8 by 52%. A more drastic inhibition (80%) of invasion was observed when S100A9 expression was reduced by siRNA.

Since migratory capacity is a prerequisite for cell invasion through the basement membrane, we next investigated the effect of siRNA for S100A8 and S100A9 on the migration of SNU484 cells by Transwell migration assay. Migration of SNU484 cells was inhibited by siRNA for S100A8 and S100A9 by 36% and 61%, respectively (Fig. 3B). The results demonstrate that S100A8 and S100A9 play crucial roles in invasive and migratory phenotypes of SNU484 cells.

### Effects of siRNA for S100A8 and S100A9 on expression of MMP-2 and MMP-9

Since tumor invasion is often associated with enhanced synthesis of MMP-2 and/or MMP-9, we next examined the involvement of S100A8 and S100A9 in MMP-2/-9 expression of SNU484 cells by gelatin zymogram assay (Fig. 4A) and immunoblot analysis (Fig. 4B). SNU484 cells expressed MMP-2 while the level of MMP-9 was undetectable, suggesting that MMP-2, rather than MMP-9, may be responsible for invasiveness of these cells. A

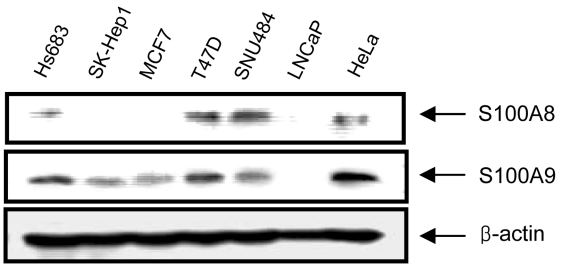


**Fig. 1.** Expression of S100A8 and S100A9 in various human cancer cell lines. S100A8 and S100A9 protein expressions were examined by immunoblot analysis with anti-S100A8 and anti-S100A9 antibodies. β-actin antibody was used as a loading control.

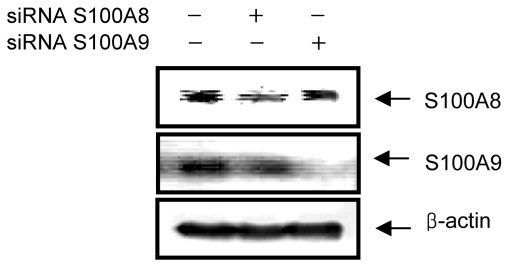


**Fig. 2.** Effects of siRNA for S100A8 and S100A9 on expression of S100A8 and S100A9 in SNU484 cells. Immunoblot analysis was performed on the cells transfected with siRNA targeting S100A8 or S100A9.Control cells were treated with Stealth™ RNAi negative control.

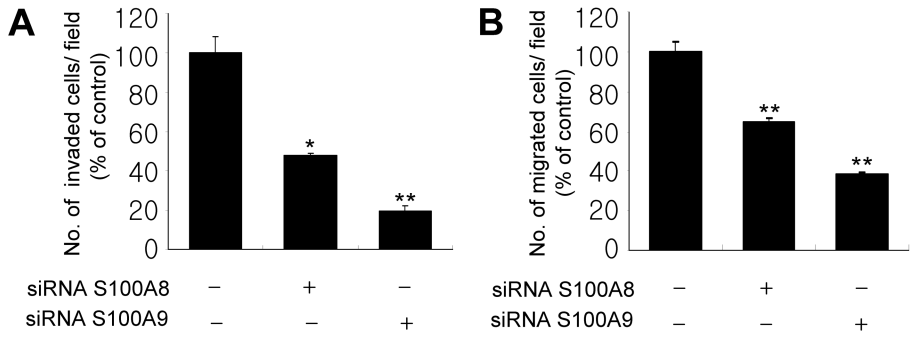


**Fig. 3**. Effects of siRNAs for S100A8 and S100A9 on invasive and migratory phenotypes of SNU484 cells. Cells were transfected with siRNAs for S100A8 and S100A9 and subjected to the *in vitro* invasion assay (A) and Transwell migration assay (B). The number of invaded or migrated cells per field was counted (×400) in thirteen arbitrary visual fields. The results represent means + S.E. of triplicates. *,**Statistically different from control at $p<0.05$ and $p<0.01$, respectively, by the two-tailed Student's *t*-test.

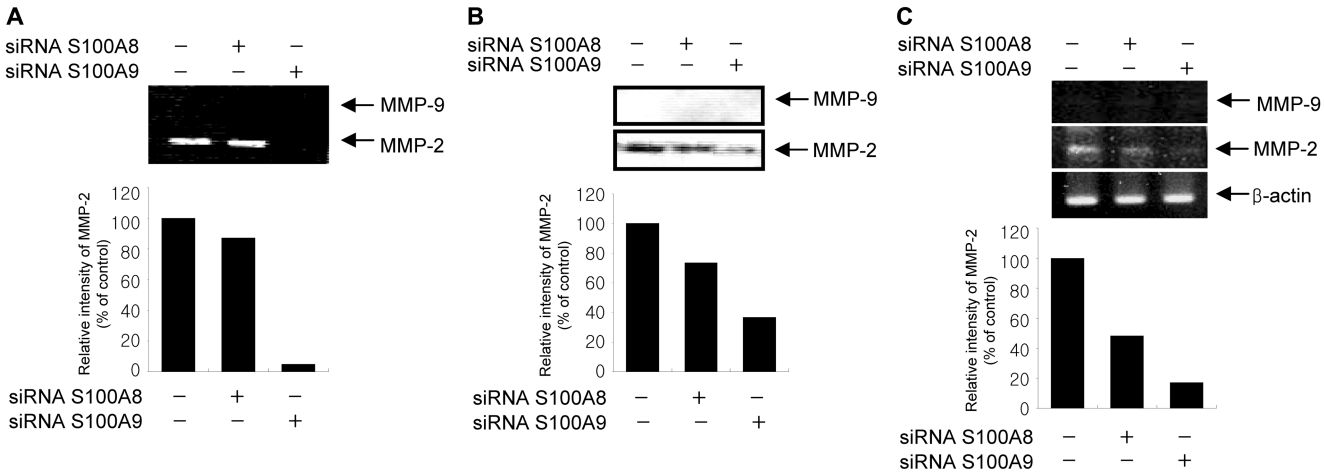


**Fig. 4.** Effects of siRNAs for S100A8 and S100A9 on MMP expression of SNU484 cells. (A) Gelatin zymogram assay was performed on the cells transfected siRNA for S100A8 and S100A9 to analyze gelatinolytic activities of secreted MMP-2 (72 kDa) and MMP-9 (92 kDa). (B) Protein expression levels of MMP-2 and MMP-9 in conditioned media were determined by immunoblot analysis. (C) The mRNA levels of MMP-2 and MMP-9 were determined by RT-PCR. Band intensities of MMP-2 were quantitated by densitometric measurements and the relative intensities of MMP-2 were plotted.

prominent decrease (92%) in MMP-2 expression was observed when S100A9 expression was inhibited by siRNA (Fig. 4A). MMP-2 expression was also inhibited by siRNA for S100A8, but to a lesser extent (11%). Similar results were obtained from immunoblot analysis (Fig. 4B), demonstrating that S100A9, and to a lesser extent S100A8, are required for MMP-2 expression in SNU484 cells.

We then investigated if S100A8 and S100A9 affected MMP-2 expression at the transcriptional level by preforming RT-PCR analysis. The mRNA level of MMP-2 was markedly decreased by siRNA for S100A9 (83%) and by siRNA for S100A9 (52%) (Fig. 4C). These results demonstrate that S100A9, and to a lesser extent S100A8, are required for transcriptional activation of MMP-2 gene in SNU484 cells.

## DISCUSSION

Overexpression of S100 proteins has been detected in a variety of human tumors, suggesting a functional contribution of S100 proteins to processes required for malignant progression including invasion and spreading of tumor cells. Our data clearly demonstrate that S100A8 and S100A9 are critical to the invasive and migratory phenotypes of SNU484 human gastric cancer cells in which MMP-2 may be involved. A more drastic inhibition of invasion, migration, and MMP-2 expression was observed by siRNA for S100A9 than by siRNA for S100A8. The data suggest that although the two proteins are essential, S100A9, rather than S100A8, exerts a major contribution to the invasive and migratory phenotypes of SNU484 cells. It cannot be ruled out, however, that these results may be due to the fact that S100A8 expression was only

partially blocked by siRNA for S100A8, while siRNA for S100A9 almost completely reduced the expression of S100A9 (Fig. 2). Further investigation would be needed to clarify the differential roles of these proteins in the invasive and migratory phenotypes and MMP-2 expression of SNU484 cells.

Although enhanced expression of S100A8 and S100A9 was observed in tissue specimens of patients with prostate cancer (Hermani *et al*., 2005), the LNCaP human prostate cancer cell line poorly expressed these proteins (Hermani *et al*., 2006). Consistently, our data showed that the LNCaP cells expressed neither S100A8 nor S100A9 (Fig. 1). Overexpression of S100A8 and S100A9 was observed in a dissected gastric cancer from a patient (El-Rifai *et al*., 2002) as well as in a gastric cancer cells line, SNU484 (Fig. 1).

Invasion is a complex process involving not only matrix-degrading protease activity but also migration through the basement. Recent reports have suggested the association of S100A8 and S100A9 with cell migration of prostate cells and phagocytes *via* calcium-dependent signals (Hermani *et al*., 2006; Vogl *et al*., 2004). It has been shown that S100A8 and S100A9 are translocated to plasma membrane in a calcium-dependent manner during monocytic differentiation (Roth *et al*., 1993). It would be worthwhile to further examine the role of calcium and S100A8/A9 in the migratory property of SNU484 cells.

Molecular mechanisms underlying the role of S100A8 and S100A9 in gastric cancer invasion, migration, and MMP-2 gene expression are currently not defined. Heterodimeric complexes of S100 proteins translocate to membranes and intermediate filaments in response to increases in intracellular calcium (Mandinova *et al*., 1998; Davey *et al*., 2000). Translocation of S100 proteins may activate signaling pathways and regulate cell motility (Mueller *et al*., 1999; Davey *et al*., 2000). One of the receptors that bind S100 proteins is the receptor for advanced glycation end products (RAGE). Blockade of RAGE suppresses tumor growth and metastasis in mice (Taguchi *et al*., 2000). RAGE signaling has been shown to contribute to the activation of signaling pathways including p38 or p44/p42 MAP kinases, cdc42/Rac and NF-κB (Hofmann *et al*., 1999; Taguchi *et al*., 2000). Our previous report shows that Rac-MKK3/6-p38 pathway is essential for MMP-2 transcriptional activation *via* activating transcription factor-2 in H-Ras MCF10A human breast epithelial cells (Shin *et al*., 2005; Song *et al*., 2006). A recent report demonstrates that S100A8 and S100A9 activate MAP kinase and NF-κB signaling pathways and trigger translocation of RAGE in human prostate cancer cells (Hermani *et al*., 2006). Further investigation will be addressed to the molecular mechanisms for the contribution of the calcium-binding proteins, S100A8 and S100A9, to MMP-2 expression and invasive/migratory phenotypes of SNU484 gastric cancer cells.

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