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Original Article

HGF-mediated S100A11 overexpression enhances proliferation and invasion of gastric cancer

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Abstract: S100 proteins are a group of low molecular weight (10-12 kDa) acidic proteins belonging to the largest family of EFhand calciumbinding proteins. S100A11, also known as S100C or calgizzarin, is an important member of the S100 family. S100A11 overexpression has been reported in a number of cancers including papillary thyroid carcinoma, colon, pancreatic, and breast cancer. One other study demonstrated that increased S100A11 expression is correlated with gastric cancer metastasis and poor overall disease prognosis. This study aimed to identify the function of S100A11 associated with cell proliferation and invasion in gastric cancer. We used cell culture, western blotting, reverse transcription-polymerase chain reaction, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, and S100A11 knock-down with short hairpin ribonucleic acid (shRNA). First, we confirmed that the S100A11 expression was upregulated by hepatocyte growth factor (HGF). The role of S100A11 was determined via knock down of S100A11. S100A11-shRNA cells showed decreased levels of metalloproteinase-9 (MMP9) and nuclear factor kappa-B (NF-κB). We also examined and confirmed the role of HGF-mediated S100A11 expression. HGF-mediated cell proliferation and *in vitro* invasion increased, and HGF-mediated apoptosis decreased in S100A11 knockdown cells. We identified the putative binding site of NF-κB in the MMP9 promoter region and confirmed its function via chromatin immunoprecipitation (CHIP) assay. Our results showed that S100A11 is upregulated by HGF through the NF-κB pathway in gastric cancer and plays a role in cell proliferation and invasion in gastric cancer. It may thus be a possible target for gastric cancer therapy.

Keywords: HGF, S100A11, invasion, gastric cancer

Introduction

S100 proteins including over 20 members, are a group of multi-gene calcium binding proteins with low molecular weight (10-12 kDa), encoded by a separate gene, and are only expressed in vertebrates; they are often overexpressed in normal tissues and human cancer tissues [1-3]. Several studies have reported that S100 proteins are involved in tumor progression. S100A2 is silenced by hypermethylation and acts as a tumor suppressor gene in lung [4], breast [5, 6], and prostate cancer [7]. S100A6 is overexpressed in many human cancers including pancreatic cancer [8, 9], malignant melanoma [10, 11], and colorectal cancer [12].

S100A11 is a less well-known protein of the S100 family. S100A11 was first discovered in chicken gizzard smooth muscle [13]. This protein is reported to exist at different levels in different tumor types. S100A11 is overexpressed in various cancer types including breast [14], prostate [15], non-small cell lung [16], and colorectal cancer [17].

However, only one study reported that S100A11 overexpression is associated with lymph node metastasis in gastric cancer cells from a surgically dissected specimen [18]. We have previously studied the gastric cancer genes upregulated by hepatocyte growth factor (HGF) treatment using a human complementary deoxyribonucleic acid (cDNA) microarray to confirm the effect HGF on gastric cancer pathogenesis using gastric cancer cell lines, NUGC-3 and MKN-28. Among the upregulated gastric cancer genes, we investigated the role of S100A11 in gastric cancer pathophysiology.

Materials and methods

Cell culture

We used two human gastric cancer cell lines: poorly differentiated adenocarcinoma, NUGC-3 and the moderately differentiated tubular adenocarcinoma, MKN-28, which were obtained from the Korea Cell Line Bank (Seoul, Korea). The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 2 mM L-glutamine, two-fold vitamin solution, and 50 U/mL penicillin/streptomycin (Life technologies, Inc., Gaithersburg, MD, USA). Unless otherwise noted, cells underwent passage and were removed from flasks when 70-80% confluent.

Northern blot analysis

Total ribonucleic acid (RNA) was extracted by acid-phenol-guanidium thiocynanate-chloroform extraction. Total RNA (10 μg) was separated on a 1% formaldehyde agarose gel and transferred to a Hybond N^+ nylon membrane by the capillary method. RNA was cross-linked by UV irradiation (1400 μJ/cm^2) using a UV Crosslinker (UVP Inc., Upland, CA, USA). The membrane was hybridized with a ^{32}P-labeled *c-fos* or *c-jun* probe overnight at 42°C and then washed in 2 × SSC for 5 min at room temperature, 2 × SSC/0.1% SDS at 42°C for 30 min, and 0.5 × SSC/0.1% SDS at 42°C for 30 min. The membranes were exposed to X-ray films at -70°C. Equal loading of the RNAs was confirmed by hybridization with a ^{32}P-labeled GAPDH probe.

cDNA microarray analysis

The cDNA microarray, containing a set of 17,448 sequence-verified human cDNA clones, was provided by GenomicTree Inc., (Daejeon, Korea). The cDNA microarray experiments were performed. Briefly, total RNA (100 μg) was reverse-transcribed in the presence of Cy3-dUTP or Cy5-dUTP (25 mM stock; NEN Life Science Products, Boston, MA, USA) at 42°C for 2 h. The labeled cDNA was then hybridized with the cDNA microarray at 65°C for 16 h. The hybridized slides were washed, scanned with an axon 4000 B scanner (Axon Instruments), and analyzed using GenePix Pro 4.0 (Axon Instruments). The raw data were normalized and analyzed using GeneSpring 6.0 (Silicon Genetics). Genes were filtered according to their intensities in the control channel. When control channel values were < 80 in all the samples, the genes were considered unreliable. Intensity-dependent normalization (LOWESS) was performed wherein the ratio was reduced to the residual LOWESS fit of the intensity versus ratio curve. Average normalized ratios were calculated by dividing the averaged normalized signal channel intensity by the averaged normalized control channel intensity. Welch's ANOVA test was performed for *P*-values ≤ 0.01 and 0.05 to identify genes in differentially expressed samples. Correlation analysis was performed using Pearson's correlation (-1 to 1). Spots showing changes of ≥ 2-fold were considered significant.

Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR)

Complementary DNA (cDNA) was synthesized from total RNA using MMLV reverse transcriptase (Promega Corp., Madison, Wi, USA) by the oligo (dT) priming method in a 10-μL reaction mixture. PCR was performed in a 10-μL reaction volume containing 10 mM Tris-HCl pH 8.5, 50 mM KCl, 1 μL cDNA, 200 μM dNTPs, 1 mM $MgSO_4$, 1 U platinum pfx Taq polymerase, and 2 μM primers. The reactions were subjected to initial denaturation at 95°C for 4 min; 27 cycles of 94°C for 15 s, 60°C for 15 s, and 72°C for 30 s; and a final extension at 72°C for 10 min. The PCR products were separated on a 1.5% agarose gel containing ethidium bromide and visualized on a UV transilluminator.

Western blot analysis

Cells were harvested and incubated with a lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Trion X-100, 10% glycerol, 1 mM PMSF, 1 mM sodium vanadate, and 5 mM NaF) with protease inhibitors and centrifuged at 15,000 rpm, 4°C for 10 min. Proteins (50 μg) were separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were soaked in 5% non-fat dried milk in TTBS (10 mM Tris-HCl pH 7.5, 150 mM NaCl, and 0.05% Tween-20) for 30 min and then incubated overnight with a primary antibody at 4°C. After washing 6 times with TTBS for 5 min, the membranes were incu-

bated with a horseradish peroxidase-conjugated secondary antibody for 1 h and 30 min at 4°C. The membranes were rinsed thrice with TTBS for 30 min and the antigen-antibody complex was detected using the enhanced chemiluminescence detection system.

Zymography

Culture supernatants were denatured in the absence of reducing agent and were electrophoresed in 10% polyacrylamide gel containing 0.1% (W/V) gelatin for metalloproteinase (MMP). The gel was incubated at room temperature for 2 h in the presence of 2.5% Triton X-100 and subsequently at 37°C overnight in a buffer containing 10 mM $CaCl_2$, 0.15 M NaCl, and 50 mM Tris (pH 7.5). The gel was then stained for proteins with 0.25% Coomassie brilliant blue solution in methanol: acetic acid: water (4:1:5) and destained in the same solution without the dye; enzyme activity was detected as negatively-stained regions. Zymographic analyses were performed in at least three independent experiments.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

The cells and S100A11-short hairpin RNA (shRNA) (1,500/well) were seeded in 96-well plates in DMEM supplemented with 5% FBS and incubated for 24 h. Cells were then serum-starved for 24 h and treated for 72 h with or without HGF (10 ng/mL). At the end of this incubation period, 50 µl of 2 mg/mL MTT solution was added and the cells were incubated for 3 h at 37°C. The supernatant was carefully removed by aspiration, and the converted dye was dissolved in 100 µL dimethyl sulfoxide. The plates were placed in a microplate shaker for 5 min, and absorbance was measured at 570 nm using a Bio-Rad multiscan plate reader.

S100A11 knock-down with shRNA

The human S100A11-specific shRNA expression vector (S100A11-shRNA, EG6282) containing a S100A11-targeted shRNA sequence (AAACCCAGGGCTGCCTTGGAAAAG) was purchased from Open Biosystems (Huntsville, AL, USA). Cells were transfected with S100A11-shRNA using Lipofectamine (Life Technologies Inc., Gaithersburg, MD, USA). Clonal selection was conducted by culture with puromycin (10 µg/mL) followed by serial dilution of the cells. Stable transfectant clones with low expression of the target genes were identified by western blot analysis.

Standard two chamber invasion assay

Control cells and transfected cells (1×10^4) were placed in the upper chamber of a Matrigel migration chamber with 0.8-micron pores (Fisher Scientific, Houston, TX, USA) in media containing 5% FBS with/without HGF (10 ng/mL). After incubation for 48 h, cells were fixed and stained using a HEMA 3 stain set (Curtis Matheson Scientific, Houston, TX, USA) according to the manufacturer's instructions. The stained filter membrane was cut and placed on a glass slide. The migrated cells were counted under a light microscope (10 fields at 200 × magnification).

Chromatin immunoprecipitation (CHIP) assay

The CHIP assay was performed using the CHIP assay kit (Upstate Biotechnology, Waltham, MA, USA) following the manufacturer's directions. Briefly, cells were fixed with 1% formaldehyde at 37°C for 10 min. Cells were washed twice with ice-cold phosphate-buffered saline containing protease inhibitors (1 mM phenylmethylsulphonyl fluoride, 1 mg/mL aprotinin, and 1 mg/mL pepstatin A), scraped and pelleted by centrifugation at 4°C. Cells were resuspended in a lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris-HCl, pH 8.1), incubated for 10 min on ice, and sonicated to shear DNA. After sonication, the lysate was centrifuged for 10 min at 13,000 rpm at 4°C. The supernatant was diluted in CHIP dilution buffer (0.01% SDS, 1% Triton X-100, 2 mM EDTA, 16.7 mM Tris-HCl pH 8.1, 167 mM NaCl, and protease inhibitors). Primary antibodies were added and incubated overnight at 48°C with rotation. The immune complex was collected using protein A/G agarose beads and washed with low salt washing buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 200 mM Tris-HCl, pH 8.1, and 150 mM NaCl), high-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 200 mM Tris-HCl, pH 8.1, and 500 mM NaCl), LiCl washing buffer (0.25 M LiCl, 1% NP40, 1% deoxycolate, 1 mM EDTA, and 10 mM Tris-HCl, pH 8.1), and finally 1 × TE buffer (10 mM Tris-HCl, and 1 mM EDTA, pH 8.0). The immune complex was then eluted using elution buffer (1% SDS, 0.1 M $NaHCO_3$, and 200 mM

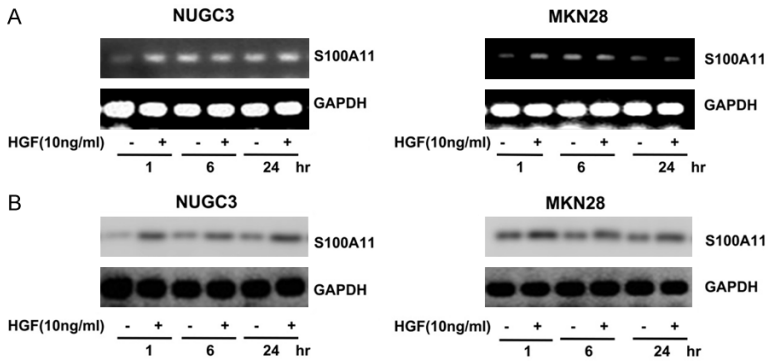

Figure 1. Overexpression of S100A11 level with HGF in NUGC-3 and MKN-28 cells. Cells were serum-starved for 24 h, treated with/without HGF (10 ng/mL) for the indicated times, and harvested. The expression levels of S100A11 RNA and protein were confirmed by RT-PCR (A) and western blot analysis (B). Representative data from three independent experiments are shown. HGF, hepatocyte growth factor; RNA, ribonucleic acid; RT-PCR, reverse transcription-polymerase chain reaction.

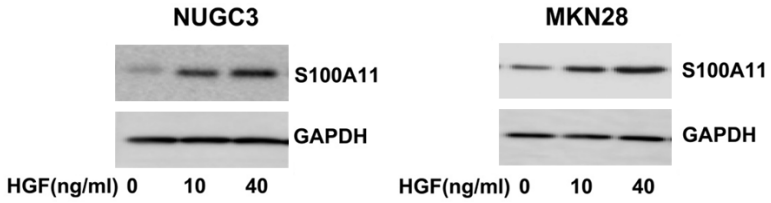

Figure 2. Dose effects of HGF on S100A11 expression in NUGC-3 and MKN-28 cells. Cells were serum-starved for 24 h, treated with HGF (0, 10 and 40 ng/ml) for 1 h, and harvested. S100A11 expression levels were confirmed by western blotting. Representative data from three independent experiments are shown. HGF, hepatocyte growth factor.

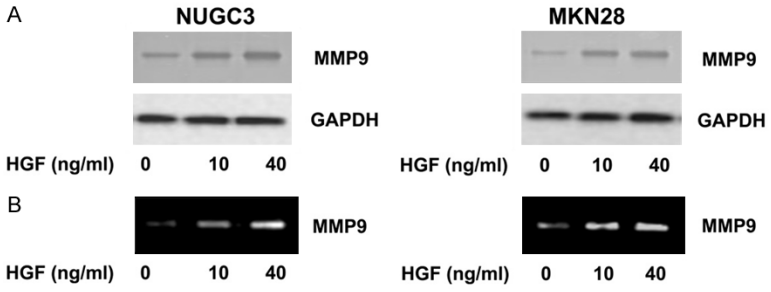

Figure 3. Overexpression of MMP9 level with HGF in NUGC-3 and MKN-28 cells. Cells were serum-starved for 24 h, treated with HGF (0, 10 and 40 ng/ml) for 1 h, and harvested. The MMP-9 protein levels in culture media were analysed by western blotting (A) and zymography (B). Representative data from three independent experiments are shown. MMP9, metalloproteinase-9.

NaCl) and the cross-links were reversed by heating at 65°C for 4 h. After the reaction, samples were adjusted with 10 mM EDTA, 20 mM Tris-HCl, pH 6.5, and 40 mg/mL proteinase K, and incubated at 4°C for 1 h. DNA was recovered and was subjected to PCR amplification of the metalloproteinase-9 (MMP9) promoter region (-980 to -754). The primers used were 5'-atggctaagattacagga-3' (forward) and 5'-acctcgggcaaatgtctt-3' (reverse).

Reagents and antibodies

Horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies were purchased from Bio-Rad Laboratories (Philadelphia, PA, USA). Recombinant human HGF (R&D systems, Inc., Minneapolis, MN, USA) and anti-S100A11 were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Antibodies against extracellular signal-regulated kinase (ERK), p38, phospho-p38, and phospho-ERK were purchased from Cell Signaling Technology (Beverly, MA, USA). PD098059 was purchased Biomol Reasearch Laboratories, Inc. (Butler Pike, PA). SB203580 and LY294002 were purchased from Calbiochem Inc. (San Diego, CA). MMP9 and nuclear factor kappa-B (NF-κB) antibodies were purchased from Santacruz (Santa Cruz, CA, USA).

Results

Up-regulation of S100A11 levels after HGF treatment

To validate S100A11 upregulation upon HGF treatment, western blot and RT-PCR analyses were performed. RT-PCR showed that the expression level of S100A11 messenger RNA (mRNA) was increased by treatment with HGF (**Figure 1A**). In addition, western blot analysis showed that the S100A11 protein level was increased at 1 h after HGF treatment (**Figure 1B**). These results confirmed that HGF

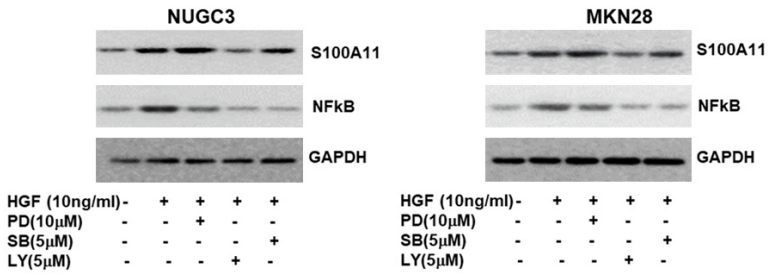

Figure 4. Effects of LY, PD, SB on the S100A11 and NF-κB expression. The treatment of LY showed the decreased level in both S100A11 and NF-κB expression level in both NUGC-3 and MKN-28 cell lines. The cells (1×10^6/well) were plated overnight in complete medium, starved for 24 h, and then treated with or without LY, PD, SB does dependent for 1 h prior to incubation with or without 10 ng/mL of HGF and harvested. The LCN2 expression was analyzed by Western blotting. This illustrates representative data from three independent experiments. NF-κB, nuclear factor kappa-B; HGF, hepatocyte growth factor.

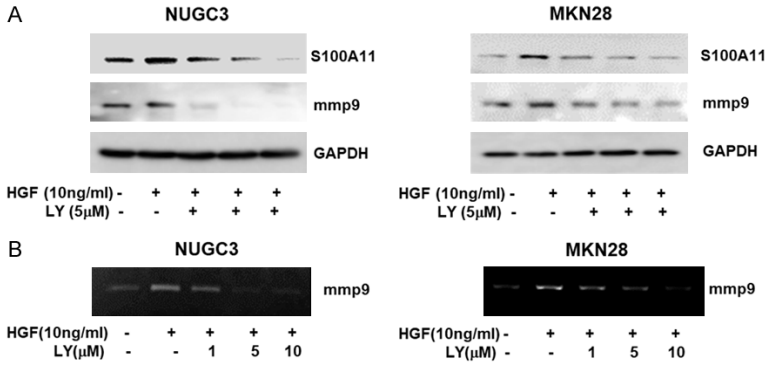

Figure 5. Down regulation of S100A11 and MMP-9 expression with LY. Cells (1×10^6/well) were plated overnight in complete medium, starved for 24 h, and then treated with or without LY at different doses for 1 h prior to incubation with or without 10 ng/ml of HGF for 48 h, and then harvested. S100A11 expression was analyzed by western blotting. MMP-9 secreted in the media was analyzed by western blotting and zymography. Representative data from three independent experiments are shown. MMP, metalloproteinase; HGF, hepatocyte growth factor.

treatment in gastric cancer cells increases the expression levels of S100A11 mRNA and protein.

Dose-dependent effects of HGF on S100A11

Dose-dependent effects of HGF on S100A11 were analyzed by western blot. S100A11 protein expression was found to be increased with increasing concentrations of HGF (0, 10 and 40 ng/mL) (**Figure 2**).

Upregulation of MMP9 levels after HGF treatment

MMP9 is well-known to have a role in cancer invasion induced by HGF. Western blot analysis and RT-PCR were performed to validate the MMP9 protein levels upon HGF treatment. The protein expression level of MMP9 was increased by HGF in a dose-dependent manner (0, 10 and 40 ng/mL) (**Figure 3A**). RT-PCR also showed that MMP9 mRNA expression increased with HGF treatment (**Figure 3B**).

Effect of LY294002, PD098059 and SB203580 on S100A11 expression

To identify whether or not phosphoinositide 3-kinase (PI-3K), ERK and mitogen-activated protein kinase activation (MAPK) is associated with HGF-induced S100A11, the cells were treated with PI3K inhibitor (LY294002) or mitogen-activated protein kinase (MEK) inhibitor (PD098059) or p38 inhibitor (SB203580) and then analyzed by Western blotting. The protein level of the HGF-induced S100A11 was decreased by LY294002. But, treatment of PD098059 and SB203580 presented no change in S100A11 expression in both NUGC-3 and MNK28 cell lines. We also analyzed the level of NF-κB with with PI3K inhibitor (LY294002) or MEK inhibitor (PD098059) or p38 inhibitor (SB203580) by Western blotting. The NF-κB protein level was decreased with SB203580 and LY294002 relatively. The treatment of LY294002 showed the decreased level in both S100A11 and NF-κB expression level in both NUGC-3 and MKN-28 cell lines (**Figure 4**).

Effect of LY294002 on S100A11 and MMP9 expression

Some studies have reported that S100 proteins regulate cell invasion and proliferation via NF-κB-dependent MMP9 signaling. To identify the pathway mediating HGF-induced S100A11 and MMP9, cells were treated with PI3K inhibi-

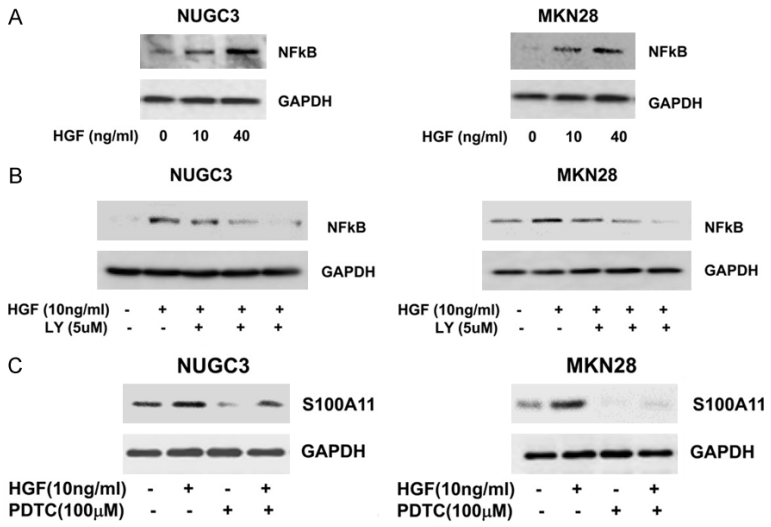

Figure 6. Effects of NF-κB on S100A11 expression. The expression level of S100A11 was decreased with PDTC treatment. Serum-starved cells were pre-treated with different doses of LY294002 for 45 min, incubated with 10 ng/ml of HGF for 15 min, and harvested. The NF-κB expression levels were confirmed by western blotting (A, B). Serum-starved cells were pretreated with/without PDTC (100 μM) for 45 min, then incubated with 10 ng/mL of HGF for 15 min, and harvested. LCN2 expression levels were confirmed by western blotting (C). Representative data from three independent experiments are shown. NF-κB, nuclear factor kappa-B; HGF, hepatocyte growth factor; PDTC, pyrrolidine dithiocarbamate; LCN2, lipocalin 2.

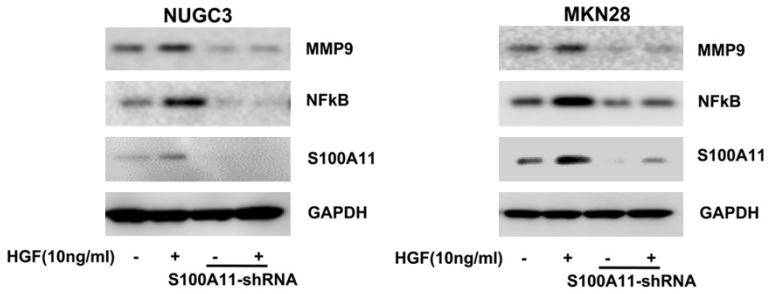

Figure 7. Down regulation of MMP9, NF-κB protein expression in S100A11 knockdown cells. Control cells and stable S100A11-shRNA cells (1×10^6/well) were plated overnight in complete medium, starved for 24 h, treated with/without 10 ng/mL HGF for 1 h, and harvested. For MMP9 analysis, control cells and stable S100A11-shRNA cells (1×10^6/well) were plated overnight in complete medium, starved for 24 h, treated with/without 10 ng/ml HGF for 48 h, and the spent medium was harvested. The expression levels of MMP9, NF-κB, and S100A11 were analyzed by western blotting. Representative data from three independent experiments are shown. MMP9, metalloproteinase-9; NF-κB, nuclear factor kappa-B; shRNA, short hairpin ribonucleic acid.

tor (LY294002) and then analyzed by western blotting. HGF-mediated S100A11 protein levels as well as HGF-induced MMP9 decreased with LY294002 (**Figure 5A**, Supplementary Figure 1). We also analyzed the expression of MMP9 upon treatment with PI3K inhibitor (LY294002) by RT-PCR; the MMP9 expression level decreased with increasing concentrations of LY294002 (0, 5 and 10 μM) (**Figure 5B**). These results indicate that HGF-induced S100A11 and MMP9 are regulated by PI3K.

Effect of NF-κB on S100A11 expression

In advance, we validated that NF-κB signaling is induced by HGF and regulated by PI3K. NF-κB expression increased with increasing concentrations of HGF (**Figure 6A**), and HGF-mediated NF-κB protein expression was decreased by the PI3K inhibitor (LY294002) (**Figure 6B**). To elucidate the regulation of S100A11 levels by NF-κB, cells were treated with NF-κB inhibitor (PDTC), and S100A11 levels were determined by western blotting. The S100A11 level was lower in PDTC-treated cells than in untreated cells in both NUGC-3 and MKN-28 cell lines (**Figure 6C**).

Effect of S100A11 knockdown on HGF-mediated MMP9

To determine the association of MMP9 and S100A11, the effect of S100A11 knockdown on MMP9 was measured. We examined the S100A11 knockdown and observed that the S100A11 level decreased in both knockdown cell lines. We measured the effect of S100A11 knockdown on HGF-mediated MMP9 expression by western blotting. The level of HGF-mediated MMP9 decreased in both S100A11 knockdown cell lines. We also measured the HGF-mediated NF-κB and found decreased NF-κB in both S100A11 knockdown cell lines. These results suggest that S100A11

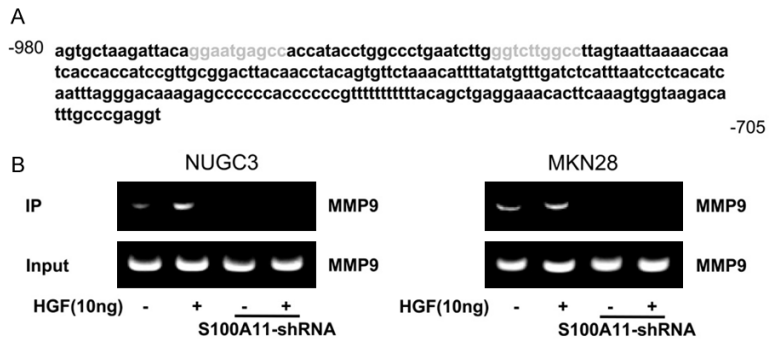

Figure 8. Effect S100A11 knockdown on MMP9 promoter activation by HGF. CHIP assay results show the amplification of a fragment of the proximal MMP9 promoter containing the NF-κB binding site but not observed the amplification in S100A11 knockdown cells. Immunoprecipitation was carried out using an anti-NF-κB antibody. Representative data from 3 independent experiments are shown. MMP9, metalloproteinase-9; HGF, hepatocyte growth factor; CHIP, chromatin immunoprecipitation; NF-κB, nuclear factor kappa-B.

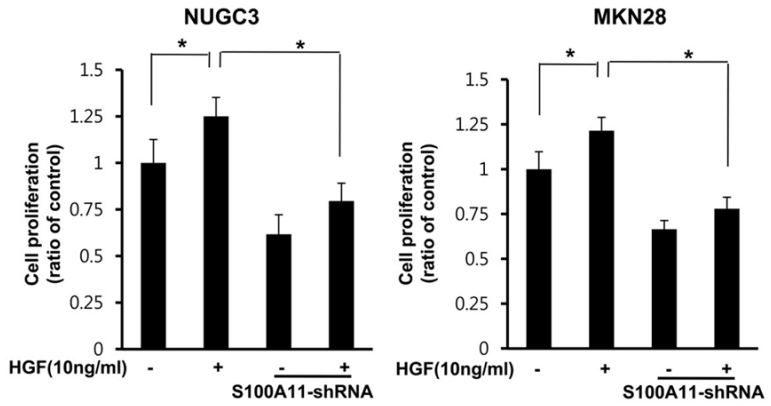

Figure 9. Down regulation of S100A11 knockdown on cell proliferation. Control cells (1,000/well) and stable S100A11-shRNA cells were seeded in 96-well plates with DMEM supplemented with 5% FBS and incubated for 24 h. After serum-starvation for 24 h, cells were treated with/without 10 ng/mL HGF for 72 h. Cell proliferation was measured using MTT assays and expressed as a percentage of HGF-untreated control cells. Values are means ± SD of three independent experiments performed in triplicate. shRNA, short hairpin ribonucleic acid; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; HGF, hepatocyte growth factor; SD, standard deviation.

may regulate HGF-mediated MMP9 expression via the NF-κB pathway (**Figure 7**, Supplementary Figure 2).

Effect of S100A11 knockdown on NF-κB binding to MMP9 promoter

To clarify whether NF-κB regulates the transcriptional activity of MMP9 mRNA by binding to the MMP9 promoter, we examined the putative binding sequence of NF-κB on MMP9 promoter using a sequence analysis program (**Figure 8A**). To examine the function of NF-κB binding activity regulated by S100A11 on the MMP9 promoter, we treated the S100-A11 knockdown cells and control cells with HGF and measured the binding activity of NF-κB to the putative biding site by CHIP assay. HGF treatment increased the NF-κB biding to MMP9 promoter, was not observed in S100A11 knockdown cells regardless of HGF treatment (**Figure 8B**). These results indicate that S100A11 may be associated with NF-κB binding to the MMP9 promoter.

Effect of S100A11 knockdown on HGF-mediated proliferation

To explore the effect of S100-A11 knockdown on HGF-mediated proliferation in gastric cancer cells, S100A11 shRNA cells and control cells were treated with HGF and cell proliferation was measured after 72 h using the MTT assay. The result showed that HGF-mediated proliferation was lower in S100A11 knockdown cells than in control cells in both gastric cancer cell lines ($P < 0.05$) (**Figure 9**).

Effect of S100A11 knockdown on HGF-mediated cell invasion

To determine whether or not S100A11 plays a role in cell invasion, we performed an *in vitro* invasion assay using Matrigel-coated migration chambers. We treated S100A11 shRNA cells and control cells with HGF and used them for the assay. After 72 h, the data showed that HGF-mediated cell invasion decreased in S100A11 shRNA cells in both cell lines ($P < 0.05$) (**Figure 10**).

Discussion

Tumor cell invasion through dense extracellular matrix is essential for cancer metastasis.

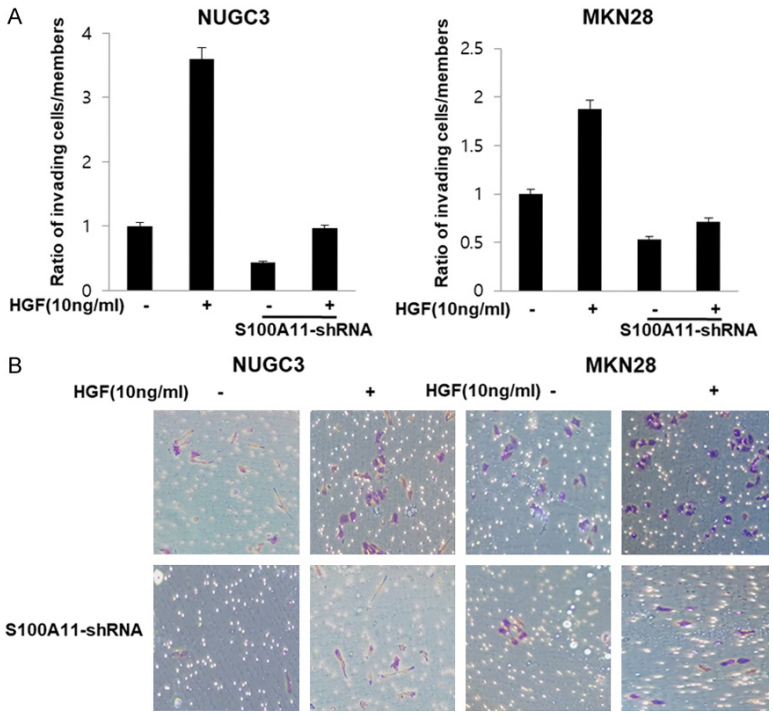

Figure 10. Down regulation of S100A11 knockdown on HGF-mediated cell invasion. Stable S100A11-shRNA cells and control cells were treated with/without 10 ng/mL HGF for 48 h. Cell invasion capacity was measured using the standard two chamber invasion assay with Matrigel-coated migration chambers. Values are means ± SD of three independent experiments. HGF, hepatocyte growth factor; shRNA, short hairpin ribonucleic acid; SD, standard deviation.

Tumor cells must have several abilities of degrading and navigating the extracellular matrix and enduring physical stress to the plasma membrane in order to disseminate from the primary cancer [13].

S100A11, a calcium binding protein, is upregulated in many cancer tissues. In contrast, some studies have also reported that S100A11 is downregulated in bladder cancer [15] and hepatocellular carcinoma [16]. Another recent report suggested the contrasting result that S100A11 protein expression promotes hepatocellular carcinoma cell invasion [19]. Thus, the role of S100A11 in cancer invasion and metastasis is not firmly known. A recent study has reported the physiologic function of S100A11 in efficient plasma membrane repair and survival of invasive cancer cells [18]. As previously mentioned, membrane degradation and repair ability is a principal factor in tumor cells for invasion and metastasis from the primary tumor and S100A11 is thought to be associated with tumor cell invasion and metastasis.

HGF plays an important role in cancer cell invasion regulated by tumor stromal interactions. The HGF/c-Met pathway plays multiple functions in cancer cells including proliferation, invasion, angiogenesis, and metastasis. In our study, we found that S100A11 was elevated after HGF treatment in a dose-dependent manner. Another study has reported that MMP2 levels decreased upon knockdown of S100A11 in laryngeal cancer cells [20]. Our previous results show that HGF increases the expression of MMP9, which is associated with extra-cellular matrix degradation, in gastric cancer cell lines.

MMPs are proteolytic enzymes that can regulate the tumor microenvironment. MMPs are known to be upregulated in almost every type of human cancer with respect to expression in normal tissues, and their high expression is associated with poor prognosis [17].

We assumed that S100A11 upregulation by HGF is also associated with MMP9 in the gastric cancer cells and investigated this association at a molecular level. Our study showed that MMP9 expression decreased upon S100A11 knockdown. We also observed that S100A11 induced by HGF regulates MMP9 via the PI3K/NF-κB pathway via decreased levels of NF-κB and MMP9 in S100A11 knockdown gastric cancer cells. In addition, S100A11 knockdown inhibited proliferation and invasion in gastric cancer cells with respect to control cells.

S100A11 has been reported to act via the epidermal growth factor receptor/AKT signaling pathway in several cancers including hepatocellular carcinoma [19], renal cell cancer [21], and laryngeal cancer [22]. However, S100A11 induction associated with HGF treatment has not been reported. Our study showed that HGF increased the NF-κB binding activity at the MMP9 promoter in control cells, but not in

S100A11 shRNA cells regardless of HGF treatment. This indicates that S100A11 affect NF-κB binding to MMP9 promoter upon HGF treatment, either directly or indirectly. However, the exact role of S100A11 protein in HGF-induced MMP9 expression was not identified. We also did not identify the pathway by which HGF regulates S100A11 expression. Additional studies are thus warranted to determine the role of S100A11-associated MMP9 in gastric cancer proliferation and invasion. Our results need to be validated by additional studies including *in vivo* experiments with knockout mice.

In conclusion, our results indicate that upregulation of S100A11 by HGF increased the level of MMP9 via the NF-κB pathway and that high S100A11 levels may enhance gastric cancer proliferation and invasion. More studies are needed to identify the mechanism by which S100A11 is regulated and how S100A11 could be used as a therapeutic target in gastric cancer.

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Disclosure of conflict of interest

None.

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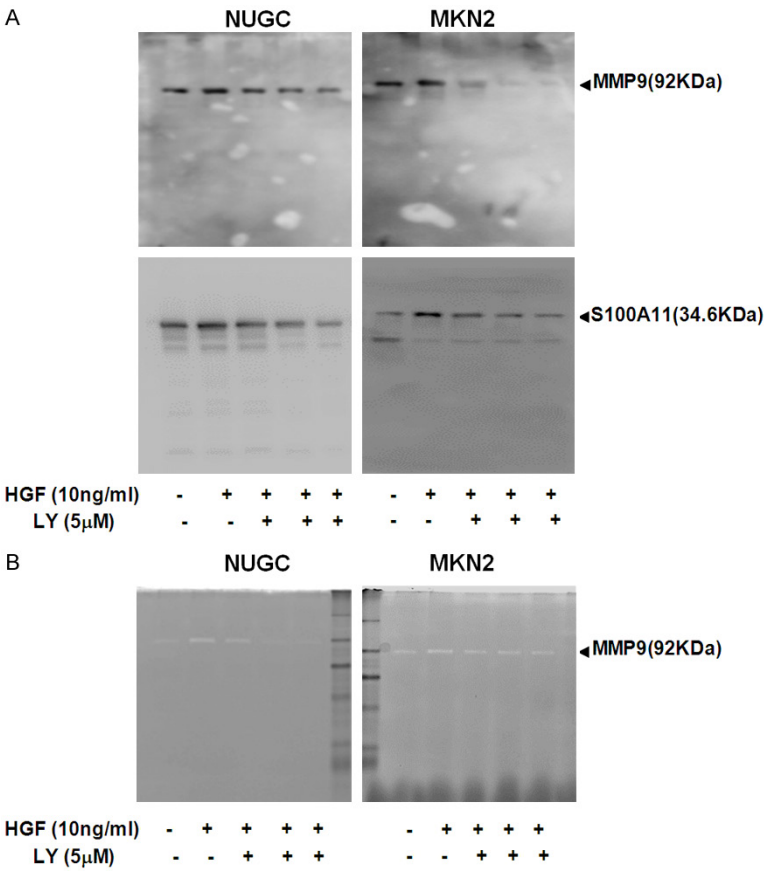
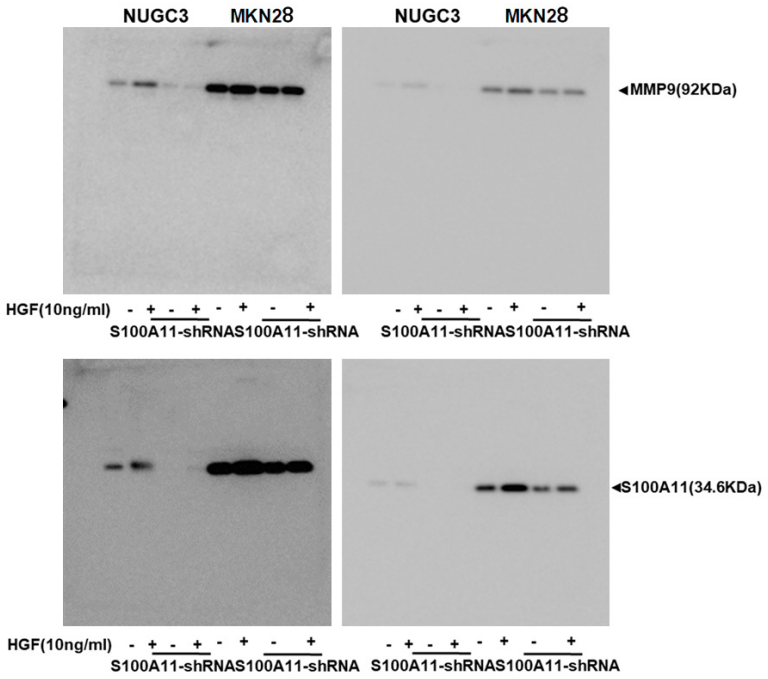


Supplementary Figure 1. Western blot whole image of **Figure 5A**.



Supplementary Figure 2. Western blot whole image of **Figure 7**.