Molecules and Cells

Validation of Neurotensin Receptor 1 as a Therapeutic Target for Gastric Cancer

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Gastric cancer is the fifth most common type of malignancy worldwide, and the survival rate of patients with advanced-stage gastric cancer is low, even after receiving chemotherapy. Here, we validated neurotensin receptor 1 (NTSR1) as a potential therapeutic target in gastric cancer. We compared NTSR1 expression levels in sixty different gastric cancer-tissue samples and cells, as well as in other cancer cells (lung, breast, pancreatic, and colon), by assessing NTSR1 expression via semi-quantitative real-time reverse transcription polymerase chain reaction, immunocytochemistry and western blot. Following neurotensin (NT) treatment, we analyzed the expression and activity of matrix metalloproteinase-9 (MMP-9) and further determined the effects on cell migration and invasion via wound-healing and transwell assays. Our results revealed that NTSR1 mRNA levels were higher in gastric cancer tissues than non-cancerous tissues. Both of *NTSR1* mRNA levels and expression were higher in gastric cancer cell lines relative to levels observed in other cancer-cell lines. Moreover, NT treatment induced MMP-9 expression and activity in all cancer cell lines, which was significantly decreased following treatment with the NTSR1 antagonist SR48692 or small-interfering RNA targeting NTSR1. Furthermore, NT-mediated metastases was confirmed by observing epithelial-mesenchymal transition markers SNAIL and E-cadherin in gastric cancer cells. NT-mediated invasion and migration of gastric cancer cells were reduced by NTSR1 depletion through the Erk signaling. These findings strongly suggested that NTR1 constitutes a potential therapeutic target for the inhibition of gastric cancer invasion and metastasis.

Keywords: gastric cancer, MMP-9, neurotensin, NTSR1

INTRODUCTION

Gastric cancer is the fifth most common type of malignancy worldwide after lung, breast, colorectal, and prostate cancers. The majority of gastric cancer cases occur in developing countries (70%; 677,000 cases), with Eastern Asian populations showing a high prevalence of gastric cancer and representing approximately half of the total cancer incidence in this area. Gastric cancer is also the third leading cause of all cancer-related deaths in both sexes worldwide (Ferlay et al., 2015). Gastric carcinoma can be the outcome of atrophic gastritis, *Helicobacter pylori* infection, intestinal metaplasia, or dysplasia (Correa, 1996).

The survival rate of patients with advanced-stage gastric cancer is low, even after receiving chemotherapy treatment. Therefore, a better therapeutic target capable of interfering with cancer-cell-signaling cascades involved in cell proliferation, metastasis, and survival is needed. The most common drugs currently used for treating gastric cancer are fluoropyrimidines, platinum compounds, anthracyclines, irinotecan, and taxanes (Wagner et al., 2006); however, the primary

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molecular prognostic factors have not yet been identified due to a general lack of knowledge regarding the molecular biology and mechanisms associated with gastric cancer. Recently, treatment with a human epidermal growth-factor receptor 2 (HER2) antibody (trastuzumab) improved overall survival in patients with metastatic gastric cancer and HER2-positive cancers (Bang et al., 2010). However, the frequency of overexpressed HER2-positive gastric cancer is relatively low and variable (4-53%; mean: 18%) (Abrahao-Machado and Scapulatempo-Neto, 2016); therefore, the introduction of new therapeutic targets for either small molecules or biologics is urgently needed. Neurotensin (NT) is an important agent that influences the growth of normal and neoplastic tissues and acts as a paracrine and endocrine hormone to modulate the digestive tract (Carraway and Plona, 2006; Evers, 2006). NT binds to G-protein-coupled receptors that transactivate epidermal growth-factor receptor and protein kinase C (PKC), followed by activated PKC promoting activation of extracellular signal-regulated kinase (ERK) pathways (Guha et al., 2002; Muller et al., 2011). NT also promotes cell proliferation and survival via activation of Akt and nuclear factor-κB (Bakirtzi et al., 2011).

NT is an important regulator of the Epithelial-mesenchymal transition (EMT) process and, consequently, cancer-cell migration, invasion, and metastasis (Zhao and Pothoulakis, 2006). Metastasis is considered the major cause of cancer-related death, with key metastatic events involved in degradation of the tissue matrix, entry of cancer cells into blood circulation, and cell invasion into diverse tissues. Matrix metalloproteinases (MMPs) are a large family of proteinases that play vital roles in cancer development and progression, including migration, invasion, and metastasis. Among MMPs, MMP-9 and MMP-2 specifically play critical roles in cancer-cell invasion (Sier et al., 1996; Sillem et al., 1999). MMP-9 expression is elevated in patients with pancreatic cancer, hepatocellular carcinoma (Maatta et al., 2000), and non-small-cell lung cancer (Zheng et al., 2010), and overexpressed MMP-9 is observed in both prostate cancer and breast cancer cells (Aalinkeel et al., 2011; Leifler et al., 2013). In gastric cancer cells, MMP-9 expression can be induced by stimulation with claudin-4 and bone morphogenic protein through the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/Akt and ERK pathways to promote cell invasion and metastasis (Hwang et al., 2014; Kang et al., 2010). Moreover, MMP-9 activation is reportedly mediated by NT expression via the mitogen-activated protein kinase (MAPK)/ERK pathway (Akter et al., 2015).

We previously found that plasma NT levels were significantly elevated in plasma samples of gastric cancer patients relative to those observed in normal human samples. The specificity and sensitivity associated with plasma NT as a gastric cancer marker indicated that it might be a strong candidate as a gastric cancer diagnostic marker (Akter et al., 2015). In this study, we tested the hypothesis that NTSR1 plays important roles in gastric cancer progression and could serve as new specific and effective therapeutic target. Here, we validated NTSR1 as a therapeutic target in gastric cancer by measuring *NTSR1* mRNA levels in gastric cancer cells and human tissue samples. Additionally, we evaluated the signaling mechanisms associated with NTSR1-mediated MMP-9 activation in various gastric cancer cell lines, as well as those of other cancers.

MATERIALS AND METHODS

Human gastric cancer samples and cell lines

A total of 60 frozen gastric cancer samples were obtained from the Chonnam National University Hwasun Hospital (Hwasun, Korea) and supported by the Ministry of Health, Welfare, and Family Affairs. Informed written consent was obtained from all subjects according to the Declaration of Helsinki, and the study was approved by the Institutional Review Board of the Catholic University of Korea, College of Medicine (MC15SISI0015). Tissue sample information is summarized in Table 1. There was no evidence of familial cancer in any of the patients. The human gastric cancer cell lines MKN-1, MKN-45, AGS, SNU-1, SNU-5, and SNU-16, and other cancer-cell lines, including A549 (lung cancer), MDA-MB231 (breast cancer), PANC-1 (prostate cancer), and SW480 (colon cancer), were obtained from the Korea Cell-line Bank (KCLB, Korea).

Table 1. Characteristic features of gastric cancer tissue sample

Parameters	Patients
Gender (n)	
Male	45
Female	15
Median age (years)	64 ± 12
Cancer properties	
Depth of wall invasion (n)	
T1	5
T2	13
T3	19
T4	23
Lymph node metastasis (n)	
N1	18
N2	11
N3	14
N4	17
Differentiation (n)	
Poor	26
Moderate	25
Well	9
Cancer stages (n)	
Stage I	9
Stage II	16
Stage III	35
Stage IV	0
Lauren's classification (n)	
Diffuse	26
Intestinal	34

Patient's sample information are categorized by sex, age and cancer properties.

MMP-9 activity assay and enzyme-linked immunosorbent assay (ELISA)

Gastric and non-gastric cell lines were treated with or without NT (Peptron, Inc., Korea), followed by treatment with SR48692 (Sigma-Aldrich, USA) and transfection with NTSR1 small-interfering RNA (siRNA). MMP-9 activity in gastric cancer cells and other cancer-cell lines was measured based on fluorescence resonance energy transfer assay. After activation of pro-MMP-9 by p-amino mercuric acid, an internally quenched substrate (Sigma-Aldrich, USA) of 7-methoxycoumarin-4-acetyl-Pro-Leu-Gly-Leu-β-(2,4-dinitrophenylamino) Ala-Arg amide was used for the experiment. Cleavage of the substrate by active MMP-9 gives rise to fluorescence, which can be measured using a fluorescence spectrometer (excitation = 328 nm; emission = 393 nm). The MMP-9 activity is directly proportional to the fluorescence level obtained (Hasan et al., 2016). MMP-9 levels in cell-culture media were analyzed using the MMP-9 ELISA kit from Abcam (UK). Cell-culture supernatant was collected and centrifuged at 10,000 g for 10 min at 4℃ to eliminate cell debris, followed by transfer of the supernatant to the MMP-9 ELISA plate according to manufacturer instructions. The relative MMP-9 level for each sample was determined by dividing by the total protein content of the whole-cell extract.

Protein extraction and western blot

Protein extraction and western blot analysis of selected proteins were performed using cell extracts according to previously described methods (Akter et al., 2015). Briefly, after harvesting the cells using trypsin-ethylenediaminetetraacetic acid (EDTA; Life Technologies, USA) and washing twice with phosphate-buffered saline (PBS), TNN-EDTA lysis buffer supplemented with a protease-inhibitor cocktail was added, and equal amounts of protein from each sample were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using a 10% polyacrylamide gel, followed by transfer onto nitrocellulose membranes (Pall Corporation, USA). Membranes were blocked with PBS containing 5% non-fat dry milk and 0.2% Tween 20 and blotted overnight using primary antibodies. The blots were than incubated with the appropriate secondary antibodies for 1 h at room temperature, followed by visualization of the bands using a western blot luminal reagent from Santa Cruz Biotechnology (USA).

Wound-healing assay

Confluent cells in six-well plates were starved in serum-free media for 24 h. A sterile 200-μL pipette tip was then used to initiate a wound by creating a scratch on the cell monolayer, and cell debris was removed by washing twice with growth medium. The culture medium was replaced with growth medium [5% fetal bovine serum (FBS)] containing NT, and NTSR1 siRNA. Cells were incubated for 24 h, followed by image collection at a magnification of 100×. This assay was performed in triplicate.

Matrigel-invasion assay

Cell invasion was assessed using transwell chambers with an 8-μm pore size. Cells (1×10^5 cells/well) were suspended in 100 μl of RPMI-1640 serum-free medium and added to the upper chamber that had been precoated with Matrigel (BD Bioscience, USA), followed by the addition of serum-free RPMI-1640 medium containing NT or NT+ NTSR1 siRNA. The lower cavity of the transwell was filled with 600 μl of 10% FBS containing fibronectin (5 μg/ml) as a chemoattractant. After incubation in a humidified incubator for 24 h, cells in the upper chamber were removed with a cotton swab. Cells in the bottom chamber were fixed with 4% formaldehyde and permeabilized with 100% methanol, followed by staining with Giemsa (Sigma-Aldrich, USA) for 10 min. Cells were counted using a fluorescence microscope (Nikon eclipse TE 2000-U). Each experiment was performed in triplicate.

siRNA transfection

All cells were cultured in six-well plates. Confluent cells were transfected with three different NTSR1 siRNAs [si1 (sense: CGUAGGUAGGGACACGUGU[dTdT], antisense: ACACGUG UCCCUACCUACG[dTdT]); si2 (sense: CUCAGACUAAUGGAU GGUU[dTdT], antisense: AACCAUCCAUUAGUCUGAG [dTdT]); and si3 (sense: GAGUUGACGGGUUCCUUGA[dTdT], antisense: UCAAGGAACCCGUCAACUC[dTdT])] or control siRNA using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer instructions. After a 6-h incubation at 37℃, 1 ml of RPMI-1640 medium containing 10% FBS was added. After 72 h, the cells were washed in PBS, and the protein lysate was prepared for further experiments.

Immunocytochemical analysis

Immunofluorescence staining was performed after fixing the cells in 4% formaldehyde for 20 min, and nonspecific binding was blocked with Abdil solution (0.1% Triton X-100 and 2% bovine serum albumin) for 30 min. Cells were then incubated with the anti-NTSR1 antibody Santa Cruz Biotechnology (USA) in blocking buffer overnight at 4℃. After washing with Tris-buffered saline, the cells were then incubated with secondary antibodies in blocking buffer for 2 h at room temperature. After staining with 4′,6-diamidino-2-phenylindole, coverslips were mounted, and images were obtained with 40× magnification using a confocal laser-scanning microscope (Carl Zeiss, Germany).

Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was isolated and purified from noncancerous gastric mucosal and gastric cancer tissues, as well as cancer cell lines, using the RNeasy kit (Qiagen, USA), and mRNAs were first reverse transcribed into cDNA using the cDNA synthesis kit (Roche, Germany). The qRT-PCR reactions were performed using a SYBR Green supermix kit (Thermo Scientific, USA). *NTSR1* mRNA was normalized against mRNA of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Sequences of the primers were as follows: *NTSR1*: sense, 5′-CAGGTCAACACCTTCATGTCC-3′ and antisense, 5′-ATGCTG AATGTGCTGTGCTC-3′; and *GAPDH*: sense, 5′-AAATCAAGT GGGGCGATGCTG-3′ and antisense, 5′-GCAGAGATGATGA CCCTTTTG-3′. Data were reported as relative quantities according to an internal calibrator using the $2^{-\Delta\Delta CT}$ method

(Pfaffl, 2001). All samples were measured in triplicate, and the mean values were used for quantification.

Statistical analysis

Densitometry analyses were performed using CS Analyzer (ATTO, Japan). Differences between two groups were done by Student's *t* tests and comparisons among multiple groups were performed using one-way analysis of variance (SigmaPlot software (v8.0; SYSTAT Software, USA). Results are expressed as the mean ± standard error of the mean (Bang et al., 2010), and differences were considered significant at $p < 0.05$.

RESULTS

Comparison of *NTSR1* expression between gastric cancer cells and other cancer-cell lines

To evaluate the specificity of NT-mediated effects, we tested the response of other cancer-cell lines outside of those associated with gastric cancer. We observed that NTSR1 expression was higher in gastric cancer cells as compared with that observed in other cancer-cell lines. To evaluate the specificity of NTSR1 expression in gastric cancer cells relative to other cancer-cell lines, we measured NTSR1 expression in gastric, breast, lung, colon, and pancreatic cancer cells by western blot and immunocytochemical analysis. Our results indicated that NTSR1 expression was elevated in MKN-1 gastric cancer cells as compared with levels observed in MDA, PANC-1, A549, and SW480 cells (Figs. 1A and 1B), and merged-stain results showed that NTSR1 expression was the highest in MKN-1 cells relative to that observed in other cancer-cell lines (Figs. 1C and 1D). Additionally, we confirmed that *NTSR1* mRNA levels were significantly increased in gastric cancer cells as compared with those observed in other cancer-cell lines, except for those associated with colon cancer (Fig. 1E). These results indicated that NTSR1 expression was specifically elevated in gastric cancer MKN-1 cells, suggesting its potential as a gastric cancer-specific marker.

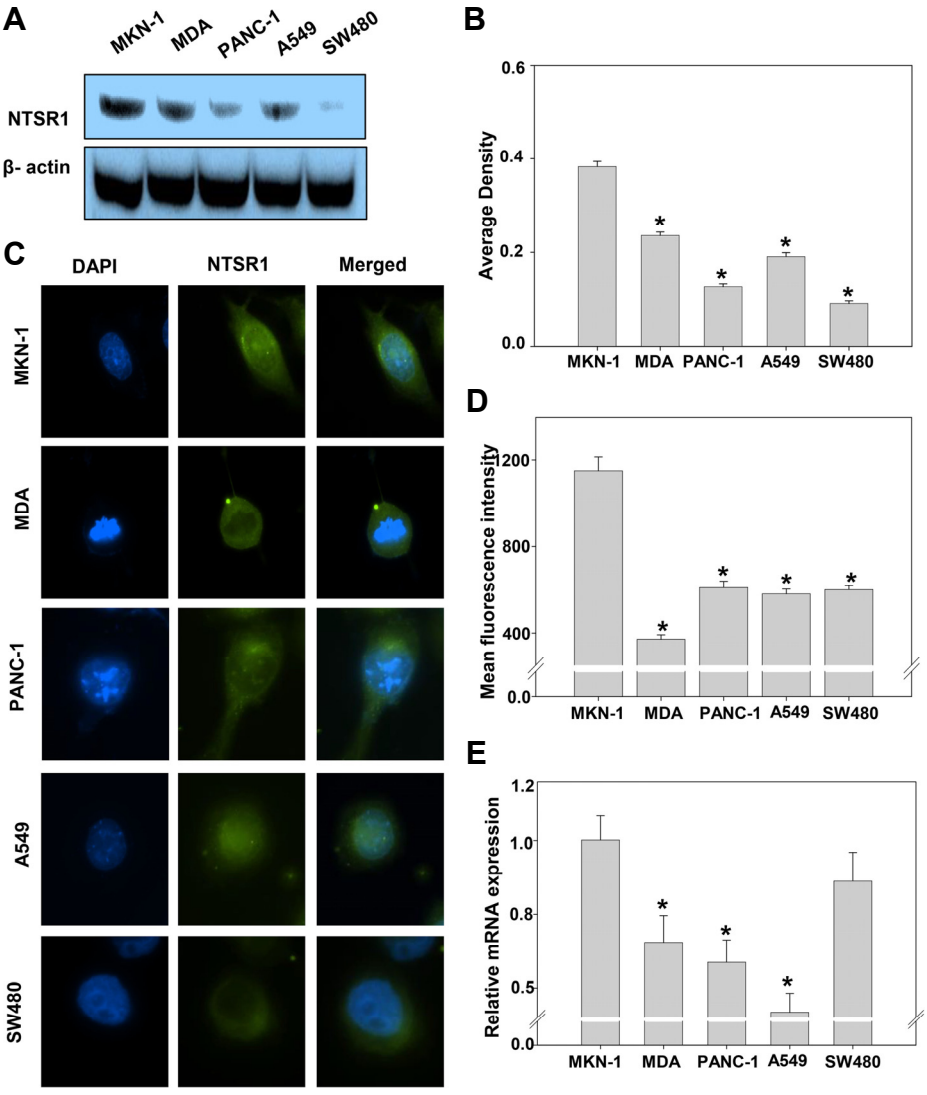

Fig. 1. NTSR1 expression in gastric cancer cells and other cancer cell lines. (A) NTSR1 expression in gastric cancer and other cancer-cell lines. (B) Bar chart shows the average densitometry analysis of western blot results. (C) Cellular localization of NTSR1 protein. (D) Bar chart showing the average fluorescence intensity of gastric cancer cells, as well as that of other cancer cells. (E) *NTSR1* expression measured using real-time qRT-PCR. $*p < 0.05$, MKN-1 vs. A549, MDA, PANC-1, and SW480 cells, respectively.

NTSR1 and NTSR2 expression during gastric cancer progression

To investigate whether NTSR1 contributes to gastric cancer progression, we performed real-time qPCR analysis in gastric cancer cell lines as well as in tissues. We analyzed *NTSR1* expression in both adherent (MKN-1 and MKN-45) and suspension (SNU-5 and SNU-16) gastric cancer-cell lines. Our results showed that *NTSR1* was overexpressed in all gastric cancer cell lines as compared with levels observed in normal gastric epithelial cells (Fig. 2A), and *NTSR1* expression in both adherent and suspension cells indicated the availability of *NTSR1* in various types of gastric cancer. However, the expression of *NTSR2* was not significant among normal gastric epithelial and cancer cells (Fig. 2B).

We further checked the expression of *NTSR1* and *NTSR2* in tissue samples. Likewise overexpression of NTSR1 detected in gastric cancer cells, we further confirmed its expression level in the gastric cancer tissue samples (Fig. 2C). However, the expression of *NTSR2* was significantly lower in gastric cancer tissue samples than normal gastric mucosa (Fig. 2D). Expression levels of *NTSR1* mRNA transcript were considerably increased in gastric cancers compared to the non-cancerous gastric mucosa ($p < 0.05$) (Fig. 2E). When we classified gastric cancers into intestinal- and diffuse-type, there was no significant difference in NTSR1 expression between intestinal- and diffuse-type gastric cancers (Fig. 2F). As expected, the mRNA expression levels of *NTSR1* were significantly higher in gastric cancers with higher TNM stage ($P < 0.05$), when compared to gastric cancers with TNM stage I and II (Fig. 2G). These results suggest that overexpression of NTSR1 may be closely associated with the progression of both intestinal- and diffuse-type gastric cancers.

Effect of NT treatment and NTSR1 knockdown on MMP-9 levels and activity in gastric cancer cells

To validate NT/NTSR1 as a therapeutic target in gastric cancer, we used six different types of gastric cancer cells, including adherent cells (MKN-1, MKN-45, and AGS) and suspension cells (SNU-1, SNU-5, and SNU-16), as well as a normal gastric epithelial cell line (HFE-145). We observed that MMP-9

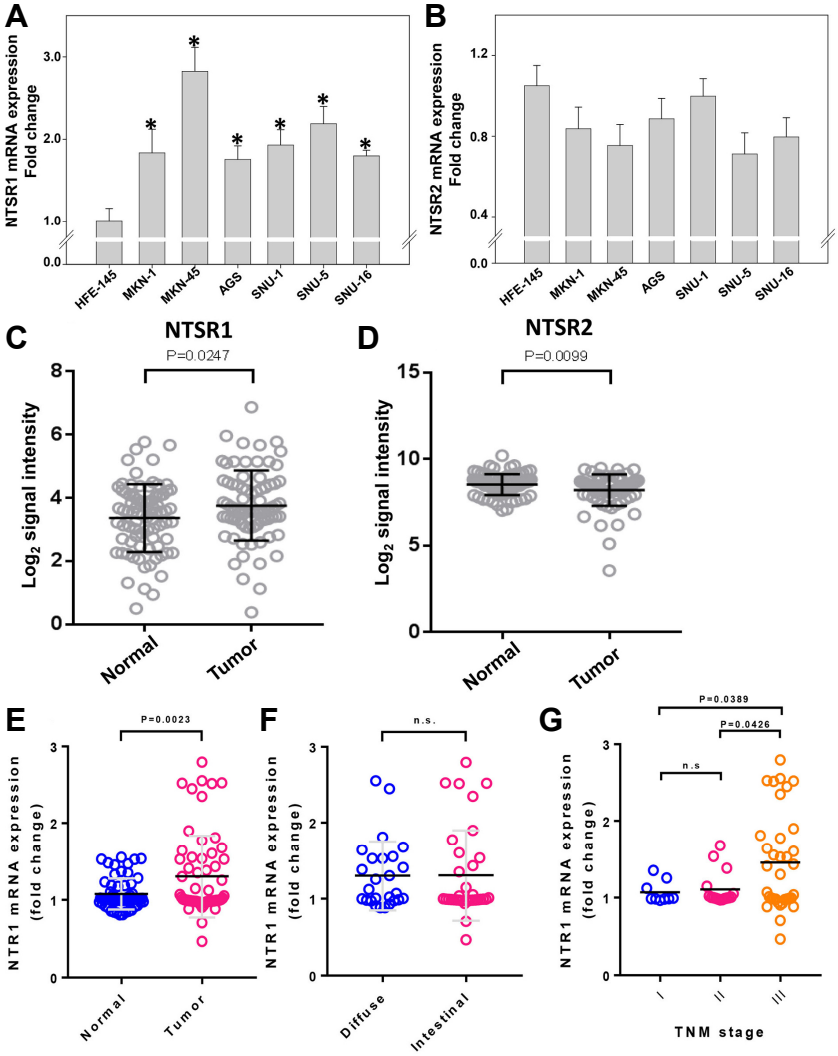

Fig 2. *NTSR1* and *NTSR2* expression in gastric cancer cells and clinical tissue samples. The ratio of (A) *NTSR1* mRNA and *NTSR2* mRNA expression normalized against GAPDH mRNA. $*p < 0.05$, gastric cancer cells vs. normal gastric epithelial cells. (C) *NTSR1 and* (D) *NTSR2 expression in gastric cancer tissues. (E)* Sixty different tissues were used to measure *NTSR1* expression. (F) Changes in *NTSR1* expression in the diffuse ($n = 26$) and intestinal ($n = 34$) gastric cancer. (G) *NTSR1* expression according to different TNM-stage gastric cancer tissues. Data are presented as the mean ± SEM.

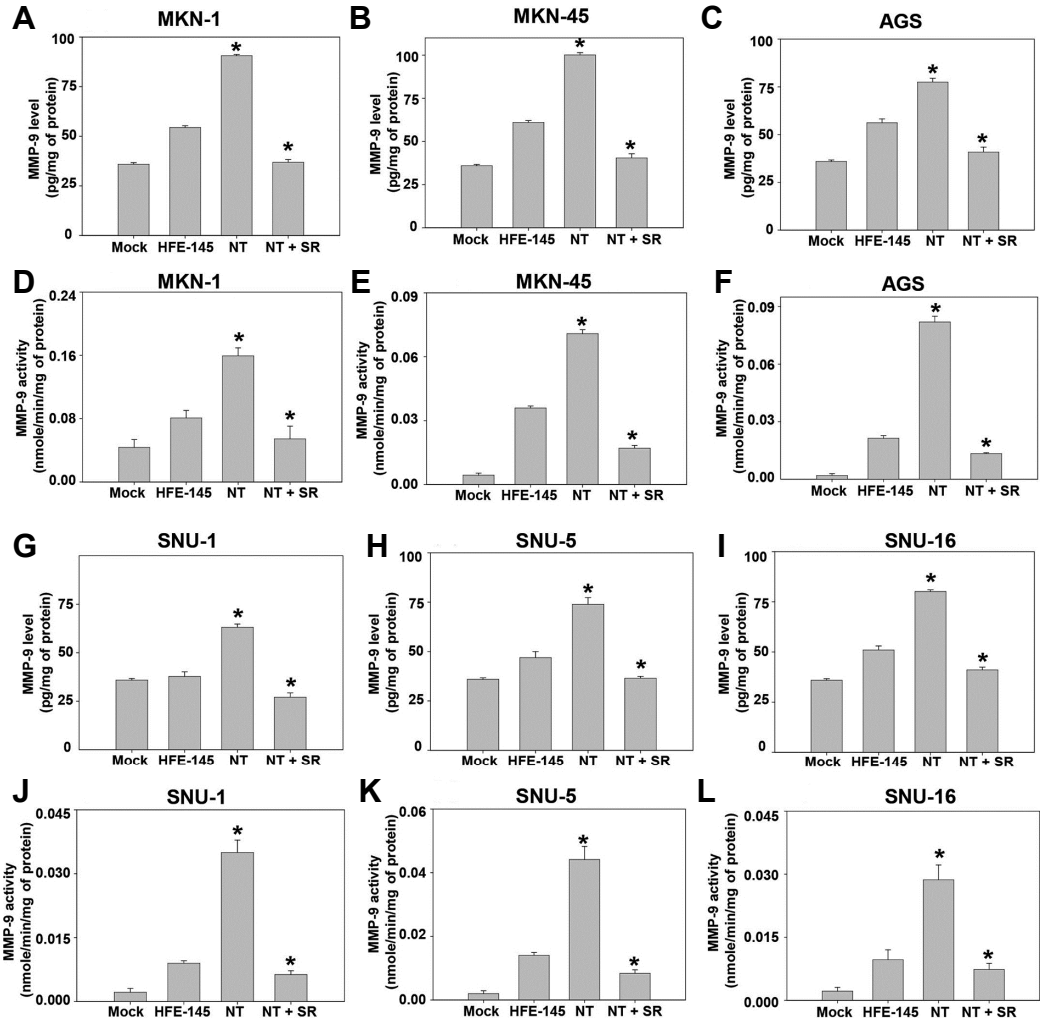

Fig. 3. NT- and SR48692-mediated expression of MMP-9 in gastric cancer cells. Quiescent adherent (A, D) MKN-1, (B, E) MKN-45, and (C, F) AGS cells and suspension (G, J) SNU-1, (H, K) SNU-5, and (I, L) SNU-16 cells were treated with NT (1 μM) for 5 min, SR48692 (10 μM) for 30 min, or well untreated. $*p < 0.05$, NT-treated cells vs. untreated and SR48692-treated cells. Results are expressed as the mean ± SEM.

expression and activity were significantly increased ($p < 0.05$) in all NT-treated gastric cancer-cell lines as compared with mock-treated and HFE-145 cells; however, when the cells were co-treated with NT and an NTSR1 antagonist, MMP-9 expression and activity were decreased relative to those observed in groups treated with only NT (Fig. 3). Additionally, *NTSR1* siRNA-transfected gastric cancer cells exhibited significant reductions in MMP-9 levels and activity as compared to levels observed in NT-treated cells (Fig. 4). These results indicated that NTR1 was required for MMP-9 expression and activity in gastric cancer-cell lines.

Effect of NTSR1 siRNA treatment on cancer-related signaling pathways

To validate NTSR1 as a gastric cancer-specific therapeutic target, as well as its role in the migration and invasion of gastric cancer cells, NTSR1 expression was knocked down in gastric cancer cells by siRNA transfection. In MKN-1 cells, NTSR1 levels were significantly reduced following transfection of si1 and si3 as compared with those observed following transfection of the negative-control siRNA or si2 (Figs. 5A and 5B).

In our previous study, we measured NT- and SR48692-mediated ERK and PI3K phosphorylation (p-ERK and p-PI3K, respectively) (Akter et al., 2015). Here, we evaluated the effect of NTSR1 on p-PI3K and p-ERK levels in MKN-1 and MKN-45 cells transfected with *NTSR1* siRNA. Transfection with *NTSR1* siRNA significantly reduced p-PI3K and p-ERK levels as compared with those observed in MKN-1 cells transfected with the negative-control siRNA (Figs. 5C and 5D). Therefore, we then determined how the subsequent downregulation of the PI3K/MAPK pathway effected MMP-9 expression and the proliferative and invasive capabilities of gastric cancer cells.

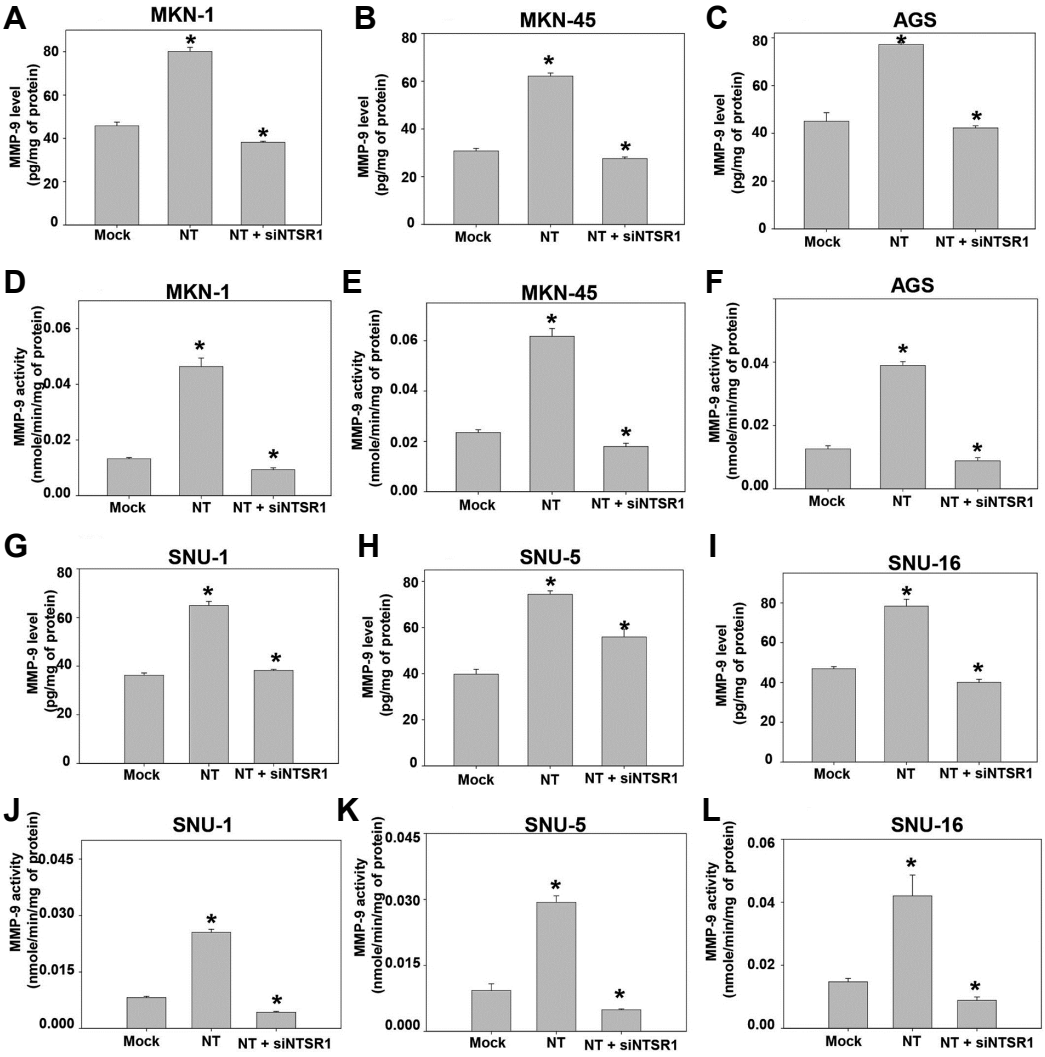

Fig. 4. Effects of *NTSR1* siRNA transfection on MMP-9 levels in gastric cancer cells. NTSR1 knockdown (si1) in confluent adherent gastric cancer (A, D) MKN-1, (B, E) MKN-45, and (C, F) AGS cells and suspension gastric cancer cells (G, J) SNU-1, (H, K) SNU-5, and (I, L) SNU-16 cells. $*p < 0.05$, NT-treated cells vs. untreated and siRNA-treated cells. Results are expressed the as mean ±SEM.

Effect of NTR1 knockdown on gastric cancer-cell migration and invasion

We then examined the expression of two significant EMT-related protein markers, E-cadherin and SNAIL, following NTSR1 knockdown. Following NT treatment, we observed that SNAIL expression was elevated, whereas E-cadherin expression was suppressed (Figs. 6E and 6F). Moreover, migration and invasion of *NTSR1* siRNA-transfected MKN-1 cells were examined by wound-healing and Matrigel-invasion assays, with cell migration measured by calculating the total percentage of the wounded area covered by the cells. We observed significantly higher numbers of cells covering the total area of wounded NT-treated cells as compared with controls. Additionally, cell migration was significantly reduced in *NTSR1* siRNA-transfected NT-treated cells as compared with that observed in cells treated with only NT (Fig. 6A and 6B). Similarly, the number of invading cells was also elevated in NT-treated cells as compared with that observed in untreated cells, whereas this number was reduced in *NTSR1* siRNA-transfected cells relative to NT-treated cells (Figs. 6C and 6D).

DISCUSSION

We previously reported the levels of plasma NT are higher in gastric cancer patients, as well as in gastric cancer cells, as compared with controls, and a positive correlation between NT and MMP-9 levels (Akter et al., 2015). Therefore, in this study, we confirmed the effects of NT on gastric cancer-tissue samples. The role of NTSR1 on metastasis in different gastric cancer cells and compared these results to the effects of similar treatment of other cancer-cell lines to determine whether NTSR1, could serve as new gastric cancer-specific therapeutic targets.

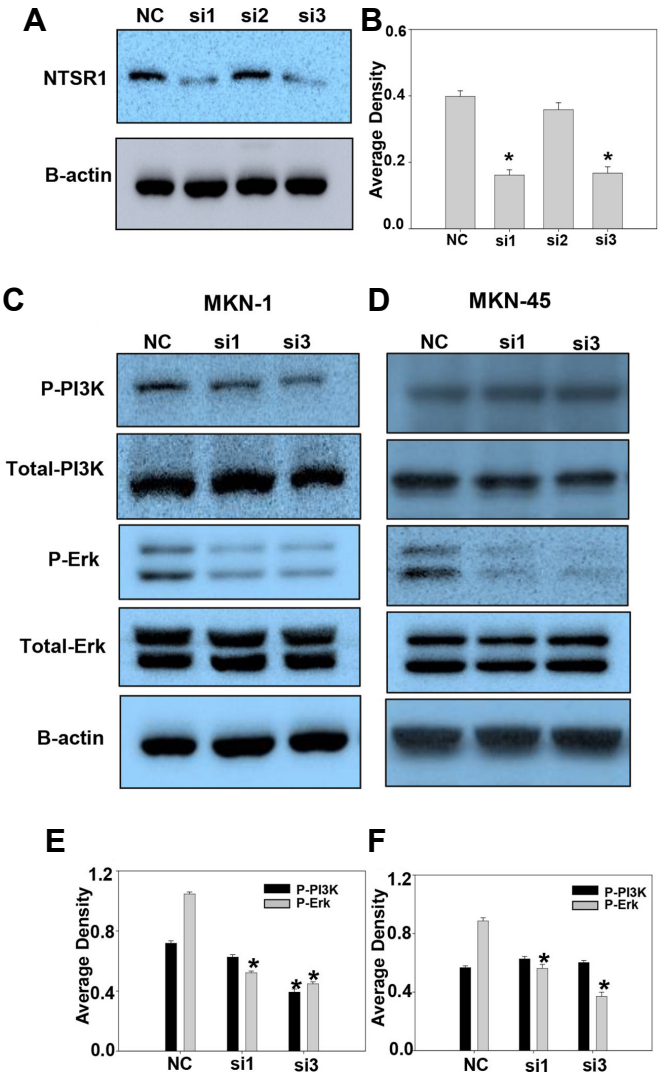

Fig. 5. *NTSR1* siRNA-mediated knockdown of proteins related to the ERK-signaling pathway in gastric cancer cells. (A) Quiescent MKN-1 cells were transfected with one of three *NTSR1* siRNAs (si1, si2, or si3). (C) siRNA-mediated depletion of NTSR1 in MKN-1 cells. (D) NTSR1 transfection in MKN-45 cells. (B), (E), (F) Bar chart shows the average densitometry analysis of western blot results. $*p < 0.05$, negative control treated cells vs. siRNA-treated cells. Results are expressed the as mean ±SEM. Results represent an example of three independent experiments.

Role of NTSR1 in gastric and other cancers

NT is a peptide located in the brain and gastrointestinal tract and functions through interactions with its receptors. Three different NT receptors have been identified (NTSR1, NTSR2, and NTSR3); however, NTSR1 and NTSR2 are members of the G-protein-coupled receptor superfamily, and their structure and function have been well-studied (Vincent et al., 1999). Here, we determined the expression of *NTSR1* and *NTSR2* in noncancerous gastric mucosa and gastric cancer tissue samples collected from gastric cancer patients. High levels of *NTSR1* expression were previously reported in breast cancer ductal and invasive compartments (Souaze et al., 2006), and here, we found that *NTSR1* was overexpressed and *NTSR2* expression was decreased in gastric cancer tissue samples as compared with levels observed in noncancerous tissue samples. Additionally, we observed that later-stage cancer was related to significantly higher *NTSR1* mRNA levels associated with gastric cancer migration and invasion. Moreover, *NTSR1* expression was confirmed in both adherent and suspension cell lines. The NT peptide and its high-affinity receptors were previously reported in small-cell lung cancer and non-small-cell lung cancer (Ocejo-Garcia et al., 2001), and NTSR expression was reported in different breast cancer, prostate cancer and colon cancer cell lines (Elek et al., 2000; Rovere et al., 1998; Seethalakshmi et al., 1997; Souaze et al., 2006). NTSR1 expression also related to worse prognoses associated with head and neck squamous cell carcinoma and ductal breast cancer (Dupouy et al., 2009; 2014; Shimizu et al., 2008). NT and NTSR1 immunoreactivity was also found in ~60% of lung adenocarcinoma biopsy specimens from patients with non-small-cell lung cancer (Alifano et al., 2010), and an NTSR1 antagonist (SR48692) inhibits the proliferation of prostate, pancreatic, and small-cell lung cancer both *in vivo* and *in vitro* (Valerie et al., 2011; Wang et al., 2011). In this study, we confirmed that NTSR1 expression was higher in various gastric cancer-cell lines as compared with levels observed in normal gastric epithelial cells, NTSR2 expression was lower in gastric cancer cell lines with normal gastric epithelial cell lines (Figs. 1 and 2).NTSR1 mRNA expression was higher in all type of gastric cancer cell

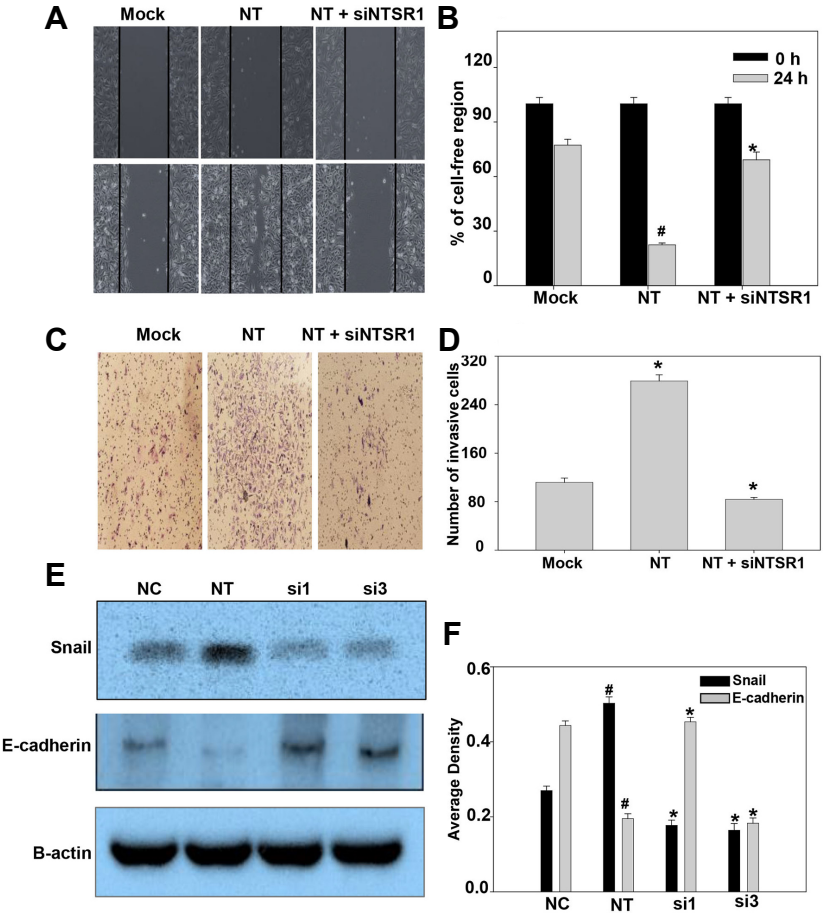

Fig. 6. EMT-marker expression and results of wound-healing and invasion assays using gastric cancer cells following NTSR1 knockdown. (A) Confluent MKN-1 cell monolayers were wounded using a pipette tip. (B) Percentage of the total area covered by the cells and the cell-free region. (C) MKN-1 cells containing serum-free media [NT- and *NTSR1* siRNA (si1)-treated cells (1×10^5 cells/well) in a 60-mm plate] were seeded in the upper chamber. After a 24-h incubation, cells that invaded the surface of the insert were stained with Giemsa stain and counted. (D) Number of invasive cells per well. (E) MKN-1 cells were transfected with *NTSR1* siRNA (si1 or si3). (F) Bar chart shows the average densitometry analysis of western blot results. Bars represent the SEM of three independent experiments. Results are expressed as the mean ± SEM. #$p < 0.05$, compared with mock and NT-treated cells. *$p < 0.05$, NT-treated cells vs. siRNA-treated cells. Results represent an example of three independent experiments.

compared with other cancer cells (Supplementary Fig. S1). Additionally, The database studies (Barretina et al., 2012) revealed a similar pattern of NTSR1 expression in stomach cancer cell lines compared with colon, prostate, lung, and breast cancer cell lines except for pancreatic cell line. In addition, all type of cancer cells (stomach, prostate, colon, breast, and pancreas) has the negative expression of NTR2 (Supplementary Fig. S2A and S2B).

NTSR1 mediated activation of MMP-9

Previous studies clarified the role of NT and NTSR1 in cancer progression using different types of cancer cells, including those from lung, breast, prostate, and pancreatic cancer (Alifano et al., 2010; Seethalakshmi et al., 1997; Souaze et al., 2006; Wang et al., 2011). Until now there is no report published that the exact function of NTSR1 in gastric cancer progression. Although recently, we reported a potential role for NT in gastric cancer through MMP-9 activation (Akter et al., 2015), and a previous study proposed *MMP-9* mRNA expression as a strong prognostic marker in gastric cancer patients (Al-Batran et al., 2012). Another study demonstrated that expression of claudin-4 promotes increased MMP-9 and MMP-2 expression in gastric cancer cells (Hwang et al., 2014). Recently, HER2 was also reported as a promising target for gastric cancer treatment; however, the frequency of HER2-overexpressing gastric cancer subtypes is >50% (Guha et al., 2002). Here, we found that NTSR1 expression in almost all gastric cancer-cell lines mediated MMP-9 levels and activity, and that NTSR1 inhibition or knockdown in both adherent and suspension cells either through treatment with an NTSR1 antagonist or siRNA inhibited MMP-9 expression and activity (Figs. 3 and 4). Therefore, these findings suggested that NTSR1-targeted treatment could be effective in a wide range of gastric cancer patients.

MMPs are upregulated in cancer cells, and MMP-2 and MMP-9 play important roles in cancer invasion and metastasis (Groblewska et al., 2012; Kim et al., 2013; Li et al., 2014). MMP-9 promotes cancer development and progression by regulating proliferation, angiogenesis, migration, and invasion. In breast cancer and head and neck squamous cell carcinoma, NTSR1 knockdown decreases cell invasion and migration (Shimizu et al., 2008). To better understand the relationship between NT and gastric cancer-cell invasion, it is important to identify the signaling pathways through which NT acts to control these activities. The Ras/Raf/ERK pathway has diverse effects on apoptosis, growth, and cell cycle arrest (Wang and Qi, 2013) and is triggered by diverse mechanisms. In this study, we observed that siRNA-mediated NTSR1 knockdown resulted in decreased p-ERK levels and downregulation of ERK-mediated signaling cascades. The

expression level of NTSR1 and its downstream signaling cascades were assumed to positively correlate. We have shown the increased expression of NTSR1 with concomitant expression of downstream pathways i.e., expression of phospho-PI3-K, phospho-ERK proteins. Moreover, their expressions were inhibited after siRNA mediated knockdown of NTSR1. If the signaling pathway is different for different gastric cancer cells it would be more promising for developing personalize therapeutic target. In the case of PI3K signaling we did not find any significant difference in NTSR1 knockdown in MKN-45 cells compared to MKN-1 cells. In our previous study, we found that the same result using NTSR1 inhibitor, where in MKN-1 cells, NT mediated downstream pathways were through ERK and PI3K signaling pathway but in the case of MKN-45 cells, ERK signaling cascade was prominent. Even though both cell lines are from human stomach cancer but according to the histological type, MKN-1 cells are from intestinal gastric cancer (IGC) and MKN-45 cells are from diffuse gastric cancer (DGC) cell lines. So we think that it can be caused by different signaling pathway for different cancer cell lines, eventhough we need more experiment for this explanation. The relative overexpression of NTSR1 and its high affinity binding with NT finally results in highly activated downstream signaling cascades (Fig. 5).

Knockdown of NTSR1 inhibits invasion and metastasis in gastric cancer

Several studies elucidated the roles of NT in carcinogenesis. One study reported direct interactions between interleukin (IL)-8 and NT, and that levels of vascular endothelial growth factor and MMP-9 were associated with co-expression of NT and IL-8 (Yu et al., 2013). NT-mediated MMP-9 expression was also observed in breast cancer cells (Souaze et al., 2006) and detected at high levels in ductal pancreatic carcinoma cells and related to tumor metastases (Pryczynicz et al., 2007). NT-mediated malignant gastric cancer involves changes in various components of the tumor microenvironment that enhance EMT, migration, invasion, and metastasis. Previous studies reported that NT-induced EMT in cancer cells via IL-8-mediated inflammatory responses, resulting in stimulation of tumor invasion and metastasis (Zhao and Pothoulakis, 2006). Additionally, in hepatocellular carcinoma cells, upregulated expression of EMT-related genes is enhanced by NT overexpression (Yu et al., 2013). MAPK-signaling cascades (Raf-1/MAPK/MEK/ERK) are regulated by IL-8 signaling and are initiated following activation of ERK, which translocate to the nucleus and upregulates the expression of SLUG, SNAIL, and TWIST and represses E-cadherin expression (Knall et al., 1996; Nagarajan et al., 2012; Venkatakrishnan et al., 2000; Weiss et al., 2012). Our results showed that ERK-mediated SNAIL and E-cadherin protein levels were regulated by NT/NTSR1 interactions to alter gastric cancer-cell invasion and metastasis (Fig. 6).

CONCLUSION

NTSR1 plays a critical role in gastric cancer invasion and metastasis through the activation of MMP-9 expression, and *NTSR1* is overexpressed in gastric cancer-tissue samples, as well as in gastric cancer-cell lines. Here, NT-mediated upregulation of MMP-9 was observed in both adherent and suspension cell lines, whereas NTSR1 inhibition following antagonist treatment or knockdown by siRNA decreased NT-mediated MMP-9 expression. Additionally, we observed NTSR1 overexpression in a wide range of gastric cancer-cell lines relative to levels observed in noncancerous cells or other cancer-cell lines. Furthermore, we showed that MMP-9 expression was dependent upon NT-mediated ERK-signaling pathways, and that NTSR1 modulated the expression of EMT markers that promote gastric cancer-cell invasion. These findings suggested that NTSR1 constitutes a potential therapeutic target for the inhibition of gastric cancer invasion and metastasis.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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