

Activation of matrix metalloproteinase-9 (MMP-9) by neurotensin promotes cell invasion and migration through ERK pathway in gastric cancer

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Abstract Neurotensin (NT) is distributed throughout the brain and gastrointestinal tract. Although the relationship between NT and matrix metalloproteinase-9 (MMP-9) activity in gastric cancer has not been reported, the elevation of MMP-9 and NT is reported in the breast, lung, prostate, and gastric cancer. The aim of our study is to investigate the relationship between NT and MMP-9 activity and the underlying signaling mechanism in gastric cancer cell lines. Commercial ELISA kits were used for estimation of NT and MMP-9 expression, and fluorescence resonance energy transfer (FRET) assay was used for measurement of MMP-9 activity. Cell migration and invasion were determined by wound healing and transwell assay. The expression of signaling proteins was measured by Western blotting. Our study reveals a positive correlation between increased plasma NT and MMP-9 activity in both of patient's serum and gastric cancer cell lines. A dose-dependent elevation of MMP-9 activity was observed by NT treatment in

gastric cancer cells (MKN-1 and MKN-45) compared to untreated gastric cancer and normal epithelial cell (HFE-145). Moreover, NT-mediated migration and invasion were observed in gastric cancer cells unlike in normal cell. The signaling mechanism of NT in gastric cancer cells was confirmed in protein kinase C (PKC), extracellular-signal regulated kinase (ERK), and phosphatidylinositol 3-kinase (PI3K) pathway. In addition, pretreatment of gastric cancer cells with NTR1 inhibitor SR48692 was shown to significantly inhibit the NT-mediated MMP-9 activity, cell invasion, and migration. Our finding illustrated NTR1 could be a possible therapeutic target for gastric cancer.

Keywords Neurotensin · NTR1 · ELISA · MMP-9 · Invasion · ERK

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Introduction

Gastric cancer (or stomach cancer) is the second leading cause of cancer death and the fourth most common malignant tumor in the world. Each year, there are about 876,000 new cases (8.7 % of the total cancer cases), and 647,000 people die from the disease (10.4 % of cancer deaths) [1]. Different risk factors for gastric cancer include foods that are smoked, salted, or pickled (nitrite or nitrite-rich food) [2], *Helicobacter pylori* infection (*H. pylori* is a bacterium that commonly infects the inner lining (the mucosa) of the stomach), long-term inflammation of the stomach, heavy smokers [3], family history of gastric cancer, and old age (>60 years) [4]. Histologically, there are two types of gastric cancer: the diffuse-type gastric cancer (DGC) and the intestinal-type gastric cancer (IGC). Genetic or epigenetic mechanisms in the gastric epithelial

stem cells and/or precursors are the cause of diffuse-type gastric cancer. Different causes of the intestinal-type gastric cancer are *H. pylori* infection, gastritis, intestinal metaplasia, dysplasia, and adenocarcinoma [5]. Until now, endoscopic evaluation was the most common method for diagnosing gastrointestinal neoplasm. However, the invasive, time-consuming, and cost-effective nature of endoscopic evaluation renders it unsuitable as a large-scale screening procedure.

The most common markers for gastric cancer detection are carbohydrate antigen 19-9 (CA19-9), α -fetoprotein antigen (AFP), pepsinogen I/II, and carcinoembryonic antigen (CEA) [6]. Plasma-soluble human leukocyte antigen-G is also a useful molecule in the differential diagnosis of colorectal, gastric, esophageal, and lung cancer when compared against healthy control [7]. The measurement of serum gastrin 17 in combination with serum pepsinogen would be beneficial in assessing the topographical pattern of atrophic gastritis and its related gastric cancer risk [8]. However, the common protein markers like CA19-9, AFP, and CEA are not sensitive enough as they are elevated only in a modest number of gastric cancer patients [6]. Sensitive biomarker establishment for gastric cancer diagnosis is still an unresolved task as the currently available markers are prone to a high degree of false negatives [6–8].

The presence of NT receptors (NTRs) in a variety of primary cancers is revealed by gene expression and Western blotting [9]. NTRs are present in gastric cancer and colorectal cancers observed by receptor autoradiography [10]. Recently, NT/NTR1 has been reported as a prognostic marker for head and neck cancers [11]. NT is a 13-amino acid neurohormone and/or neuromodulator synthesized mostly in the central nervous system and also in the gut [12]. Three known NT receptors are NTR1, NTR2, and NTR3 that bind with 8–13 amino acids in the C-terminal of NT for a similar structure–function relationship [13]. Among these receptors, the NTR1 is present in several types of cancer. In non-small cell lung cancer (NSCLC), NT and NTR1 immunoreactivity is present in approximately 60 % of lung adenocarcinoma biopsy specimens [14]. Moreover, overexpression of NTRs is observed in prostate cancer [15] and in breast cancer [16].

In Wistar rats, subcutaneous injection of NT (100–200 μ g/kg of body weight) promotes gastric carcinogenesis induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine [17]. Moreover, in a study, it was shown that out of 51 patients with invasive ductal breast cancer, 34 % of the tumors were positive for NT while 91 % were positive for NTR1 [16]. The key role of NT in the gastrointestinal tract is to regulate digestive processes in the stomach, the small intestine, and the colon. Outside the central nervous system and the brain, NT influences growth of the intestine, colon, stomach, and adrenal cortex in vivo in addition to proliferation of fibroblasts and hepatocytes [18, 19].

The principal mediators of cancer microenvironment alteration during cancer metastasis are matrix metalloproteinases (MMPs). MMPs are a Zn ion-dependent family of more than 20 enzymes well known for their roles in degrading the extracellular matrix. Among the extracellular proteinases, MMP-2 and MMP-9 play critical roles in invasion of carcinoma [20, 21]. MMP-9 is known to be involved in the development of several human malignancies, as degradation of collagen IV in the basement membrane and extracellular matrix facilitates tumor progression, including invasion, metastasis, growth, and angiogenesis [22]. MMP-9-mediated invasion was previously reported by transmembrane and secreted protein delta like-1 in lung cancer cells [23]. MMP-9 expression was also elevated in non-small cell lung cancer [24], hepatocellular, and pancreatic adenocarcinoma [25]. The overexpression of MMP-9 in prostate cancer cell lines and in breast cancer has been reported [26, 27]. It was also reported that an NT agonist can trigger MMP-9 expression in breast cancer cells [16]. However, there is no published report regarding NT-mediated MMP-9 activity in gastric cancer cells.

Bone morphogenic protein-2-mediated motility and invasion in gastric cancer cells are mediated by MMP-9 expression through the phosphatidylinositol 3-kinase (PI3K)/Akt pathway [28, 29]. Moreover, MMP-9 secretions in ovarian cancer cells are induced by the adhesion molecule fibronectin via MEK1-mitogen-activated protein kinase (MAPK) and PI3K-Akt pathways [30]. In the current study, we investigated the role and underlying signaling mechanism of NT-mediated MMP-9 activity in gastric cancer cell lines. Further, we elucidated the effects of NT-mediated invasion and migration in gastric cancer cells.

Materials and methods

Reagents and antibodies

Fluorescence resonance energy transfer (FRET) peptide, *p*-aminophenylmercuric acetate (APMA), and NT were purchased from Peptron Inc., Daejeon, South Korea. RPMI medium 1640, fetal bovine serum, 0.25 % trypsin-ethylenediaminetetraacetic acid (EDTA), and penicillin-streptomycin were obtained from Life Technologies, Grand Island, USA. Antibodies for protein kinase C (PKC), p-PKC α / β II, MAPK (extracellular-signal regulated kinase (Erk)1/2), p44/42 MAPK (Erk1/2), Akt, p-Akt (ser473), c-Raf, p-c-Raf (ser259), PI3 kinase, and p-PI3 kinase (Tyr458/Tyr199) were obtained from Cell Signaling Technology, Beverly, MA, USA. Secondary goat anti-rabbit IgG-HRP antibodies were purchased from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA. Anti-beta-Actin (N-term) antibody was obtained from Young In Frontier Co. LTD., Seoul, South Korea.

ELISA for NT in plasma

Blood samples were collected early in the morning (8–10 O'clock) from hospitalized patients after overnight fasting. After collecting blood into EDTA collection tubes, plasma was extracted by centrifuging for 10 min (10,000 rpm) at 4 °C. Collected plasma samples were stored at –70 °C until further experiments. All participants provided their written informed consent to participate in this study. We explained our study plan to all patients before receiving the informed consent. Sample collection and experiments were approved by the Institutional Review Board (IRB) of Catholic University Medical (IRB approval No. CUMC10U126). Sample information are summarized in Table 1.

Measurement of NT in the plasma of gastric cancer patients was performed using a commercially available fluorescence immunoassay kit (Phoenix Pharmaceuticals, Inc., CA, USA). This kit was designed to detect a specific peptide in the

Table 1 Characteristic features of gastric cancer patients and healthy controls participate in current experiment and diagnostic parameters of neurotensin

Parameters	Control	Patients
Male/female (<i>n</i>)	16/24	24/13
Median age (range)	43 (30–79)	58 (32–81)
Cancer properties		
Depth of wall invasion (<i>n</i>)		
T1	–	17
T2	–	5
T3	–	7
T4	–	6
Unclassified	–	2
Differentiation (<i>n</i>)		
Poor	–	23
Moderate	–	12
Well	–	2
Cancer stages (<i>n</i>)		
Stage I	–	19
Stage II	–	4
Stage III	–	9
Stage IV	–	5
IGC/DGC (<i>n</i>)	–	20/17
Neurotensin properties		
Concentration (pg/ml)	149±14	996±191*
Cutoff value (pg/ml)	237	
Specificity (%)	87.5	
Sensitivity (%)	72.9 (56–86)	

Patients and control sample information are categorized by sex, age, and cancer properties. Neurotensin level was measured by ELISA kit. Statistical significance indicated by asterisks (*) was assessed using the Student's *t* test

**p*<0.05 compared to control samples

extracted plasma using the competitive immunoassay principle. For extraction, plasma samples were acidified with an equal amount of buffer (100 % acetonitrile) and centrifuged at 12,000 rpm for 20 min at 4 °C. An Oasis hydrophilic/lipophilic-balanced (HLB) extraction column was equilibrated by washing with 1 ml of acetonitrile (once) and 1 ml of distilled water (DW) (once). Samples were then loaded slowly into the equilibrated column, slowly washed in the column using 1 ml of DW (twice), and the wash was discarded. Peptides were then slowly eluted with 50 % acetonitrile (once), and the eluent was collected into a polystyrene tube. The collected eluent was then put into a sample concentrator for 30 min at 40 °C under a stream of nitrogen. The sample concentrator passes gas over the surface of samples via stainless steel needles. After complete evaporation of the acetonitrile, the samples were put into a freeze dryer for 12–14 h. After freeze drying, each sample was reconstituted with 50 µl of 1× assay buffer (from kit).

The ELISA assay was performed next according to the manufacturer's protocols. The assay was based on highly specific antibodies for NT (human, rat, mouse) 100 %, which do not exhibit cross-reactivity with a large variety of structurally unrelated peptides (substance P, kinetensin, bombesin) 0 %. The ELISA kit had a minimum detectable concentration of 20.1 pg/ml. However, the precision (intra-assay variation) and inter-assay variation were 10 and 15 %, respectively.

Cell lines and cultures

Human gastric cancer cell lines MKN-1 and MKN-45 were obtained from Korea Cell Line Bank (KCLB, Seoul, South Korea). HFE-145 cells were obtained from the Catholic University of Korea. Cell lines were routinely cultured in RPMI 1640 media supplemented with 10 % heat-inactivated fetal bovine serum, 25 mM HEPES, and 1 % (v/v) penicillin/streptomycin. Cells were plated onto plastic culture wells of 60-mm dish plates (Corning Life Sciences, Acton, MA) at a density of 50,000 cells/cm² in the case of MKN-1 cells and 25,000 cells/cm² in the case of MKN-45 cells with serum-containing medium. The culture plates were kept at 37 °C with 5 % CO₂ in a humidified cell incubator. After 24 h, the medium was replaced with serum-free medium, and the cells were cultured for 24 h before treatment with NT.

MMP-9 ELISA assay

The protein level of MMP-9 in the supernatant of MKN-1 and MKN-45 cells was assessed by MMP-9 ELISA kit (Abcam, Cambridge, UK). After incubation with or without NT and SR48692, cell supernatant was collected and centrifuged at 10,000×g for 10 min at 4 °C to remove the cell debris. Then, supernatant was added to the MMP-9 ELISA plate, following the manufacturer's protocols. The relative protein level of

MMP-9 in each sample was determined by dividing the total protein of the whole cell extract.

Assay for MMP-9 activity

MMP-9 enzyme activity was measured in human plasma as well as in NT-treated MKN-1 and MKN-45 cells using a fluorescence method. After treatment with NT, the cells were harvested and sonicated in PBS. MMP-9 activity in the cell extract was normalized using the amount of total cellular protein. After activation of inactive MMP-9 by APMA (*p*-amino mercuric acid), an internally quenched substrate 7-methoxycoumarin-4-acetyl-Pro-Leu-Gly-Leu- β -(2,4-dinitrophenylamino)Ala-Arg amide was used for the assay. The cleavage of the substrate by active MMP-9 gave rise to fluorescence that was measured by a fluorescence spectrometer (excitation=328 nm; emission=393 nm). MMP-9 activity was directly proportional to the fluorescence level obtained [31].

Protein extraction and Western blot

MKN-1, MKN-45, and HFE-145 cells were collected using trypsin-EDTA (TE) and twice washed with PBS, then TNN-EDTA lysis buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 0.5 % Nonidet P-40 (NP40), 1 mM EDTA, and 200 mM Na₃VO₄ supplemented with protease inhibitor cocktail) were added and the cells were scraped. Lysates were incubated on ice for 40 min and centrifuged for 15 min at 4 °C to collect the supernatant. Protein concentration was measured using the bicinchoninic acid kit (Pierce), as described in the manufacturer's instruction manual. Equal amounts of proteins were separated by SDS-PAGE on 10 % polyacrylamide minigels, then transferred onto nitrocellulose membranes (Pall Corporation), blocked with PBS containing 0.2 % Tween 20 and 5 % non-fat dry milk, and blotted overnight with antibodies against phospho-PKC α/β (1:1,000), PKC (1:1,000), total PKC (1:1,000), phospho-Erk1/2 (1:1,000), total Erk1/2 (1:1,000), phospho-Akt (1:1,000), total Akt (1:1,000), phospho-c-Raf (1:1,000), total c-Raf (1:1,000), phospho-PI3 kinase (1:1,000), total PI3 kinase (1:1,000), and anti β -actin (1:5,000). Afterward, the proteins were incubated with horseradish peroxidase-labeled secondary antibody for 1 h at room temperature. The bands were visualized using Western blotting luminal reagent from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). After staining with one antibody, some blots were stripped (restored in western blotting stripping buffer, Pierce, USA) and re-probed using different antibodies for comparison and for normalization.

Wound healing assay

Cells were seeded in six-well plates and cultured until they reached confluence. Cells were starved in serum-free media for 24 h, prior to scratching with a sterile pipette tip. Wounds were scratched on the monolayer of cells using 200- μ l pipette tips. The plates were then washed twice with growth media to remove cell debris. The culture media were replaced with growth media (5 % FBS) containing NT and SR48692, and the cells were cultured for 24 h and then photographed at a magnification of \times 100. This assay was repeated three times.

Matrigel invasion assay

Cell invasion assay was performed using transwell chambers (8 μ m pore size). For the invasion assay, 1×10^5 cells/well suspended in 100 μ l serum-free RPMI 1640 were added to the upper chamber which was precoated with Matrigel (BD Bioscience, CA, USA; according to the manufacturer's protocols), and then, serum-free media containing NT and SR48692 were added. The lower chamber of the transwell was filled with 600 μ l of 10 % FBS containing fibronectin (5 μ g/ml) as a chemoattractant. After 24-h incubation in a humidified incubator at 37 °C, 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 and then finally stained with Giemsa stain for 10 min. Cells were counted using a fluorescence microscope. Each experiment was performed in triplicates.

Statistical analysis

Statistical analysis was performed using SigmaPlot software version 8.0 from SYSTAT Software Inc. (San Jose, CA, USA) and with MedCalc software (Ostend Belgium) using the *t* test for linear regression analysis. The feasibility of using NT as diagnostic markers for differentiating between gastric cancer patients and healthy controls was assessed by using the receiver-operating characteristic (ROC) curve analysis by MedCalc. Comparisons between two groups were done using the Student's *t* test. The general linear model (GLM) procedure of SAS (SAS Institute Inc., Cary, NC, USA) was used to compare among multiple groups. Results are expressed as mean \pm standard error mean (SEM). "Significant" indicates the calculated *p* is less than 0.05 (*p*<0.05).

Results and discussion

An increased NT/NTR1 expression indicates a higher rate of distant metastasis. However, there is no direct evidence for the presence of NT in plasma samples of gastric cancer. The plasma NT and its mechanism of action in gastric cancer have not

been reported yet. We found increased plasma NT and MMP-9 activity in gastric cancer patients. The underlying signaling mechanism for NT-mediated MMP-9 activity is detailed.

Plasma NT increase in cancer patients and healthy controls

Plasma NT levels were detected as 149 ± 14 pg/ml in healthy controls and 996 ± 191 pg/ml in patients with gastric cancer. The difference between healthy controls and cancer patients was found to be statistically significant using the independent sample *t* test (Fig. 1a). The abundance of plasma neuroendocrine in different cancer types has been reported in previous studies. NT and a large number of other peptide hormones have been identified with putative roles as growth factors in human cancer [32].

There were significant differences in NT level between the gastric cancer stages and the healthy controls ($p=0.001$). Disease stage-dependent variation of NT in the plasma of gastric cancer patients in the early stage (stage I) and in the progressive stages (stages II, III, IV) had significantly higher NT levels when compared to healthy controls. An early diagnosis is possible by measuring plasma NT level, as NT is significantly higher in stage I. However, there were no significant differences among different stages of gastric cancers (Fig. 1b).

The ROC curve is a fundamental tool for diagnostic evaluation to assess the performance of plasma NT in detecting

gastric cancer. The area under the curves (AUC) of healthy controls versus gastric cancer for NT was 0.88. The specificity and sensitivity of NT were found to be 87.5 and 72.9 %, respectively [95 % confidence interval (CI), 56–86]. The cutoff value of NT was 237 pg/ml (Fig. 1c). Plasma samples obtained from patients of well-differentiated cancer type had shown significantly higher NT level than poorly differentiated group. However, NT level is not affected by gender, age, depth of invasion, cancer stages, and IGC/DGC patients. A detailed sample information and NT quantification level was given in Table 1. The pre-surgery serum level of CA 19-9, CEA, and CA 72-4 was analyzed in gastric adenocarcinoma and in endoscopically diagnosed benign tumors. ROC curve analysis reported the sensitivities of CA 72-4, CEA, and CA 19-9 as 59.8, 21, and 26.3 %, respectively. The AUC was 0.86, 0.52, and 0.58 for CA 19-9, CEA, and CA 72-4, respectively [33].

Correlation between plasma NT and MMP-9 activity

MMPs, particularly MMP-9, have been shown to be one of the key enzymes in the invasion and metastatic cascade of gastric cancer. There were some previously published reports suggesting overexpression of MMP-9 levels in gastric carcinoma tissue samples [34]. By gene microarray analysis, the increase expression of MMPs was also confirmed, including MMP-2 and MMP-9 and VEGF in gastric carcinoma [35].

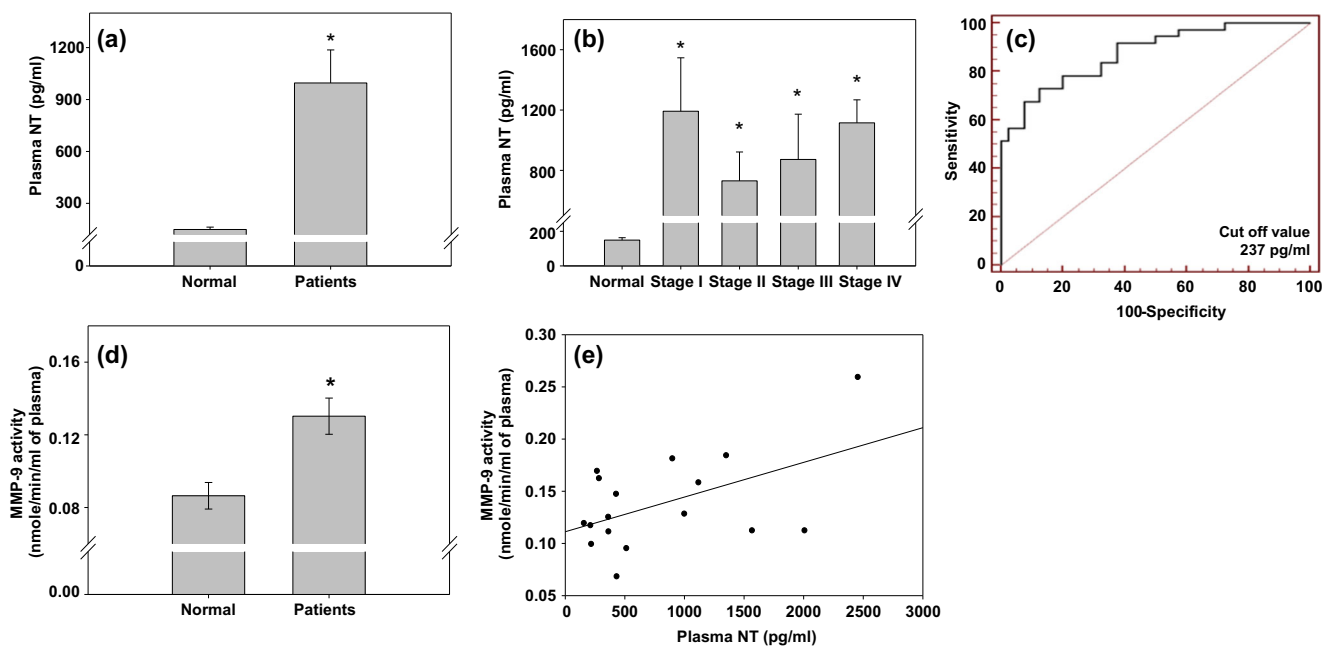


Fig. 1 NT level and MMP-9 activity in plasma samples of gastric cancer patients. **a** NT concentration in plasma samples of healthy controls and gastric cancer patients measured by commercially available ELISA. $*p<0.001$ versus control samples. **b** Disease stage-dependent variation of NT in plasma samples. $*p<0.05$ versus controls. **c** Receiver-operating characteristic (ROC) curve analyses assessing the performance of NT levels in diagnosing gastric cancer. The area under curve (AUC) for NT

was 0.88. **d** MMP-9 activity measured in gastric cancer plasma samples using fluorescence spectrometer (excitation=328 nm; emission=393 nm). MMP-9 activity is directly proportional to the fluorescence level obtained. $*p<0.05$ versus control samples. **e** Significant positive correlation between NT (*X*-axis) and MMP-9 activity (*Y*-axis) in gastric cancer patient's plasma samples. All results are expressed as mean \pm SEM

Based on our proposed hypothesis, as NT modulates MMP-9 activity, we analyzed both NT and MMP-9 activity in the same plasma samples. We found that MMP-9 activity in the plasma samples from gastric cancer patients was significantly higher ($p < 0.0004$) than in control plasma samples (Fig. 1d). MMP-9 was found to be a potent diagnostic marker for gastric cancer. Based on our observations, we hypothesized that there was a correlation between plasma NT and MMP-9 activity. Interestingly, we observed a positive correlation between plasma NT and MMP-9 activity ($r = 0.90$) (Fig. 1e). The expression of MMP-9 has been reported in other cancers [36–39]. The activation of CXCL12/CXCR4 axis in prostate cancer cells induces bone metastasis through overexpression of MMP-9 [36]. In tracheal smooth muscle cells, TNF- α induces overexpression of MMP-9 [37]. In hepatocellular carcinoma, x protein of the hepatitis B virus was reported to induce MMP-9 expression [38]. However, radiation-induced invasive properties in hepatocellular carcinoma cell lines were found to have triggered MMP-9 expression [39]. In our current study, we, for the first time, have shown a correlation between NT and MMP-9 in plasma samples from gastric cancer patients.

NT-mediated MMP-9 expression and activity in vitro

Based on the positive correlation between NT and MMP-9 in plasma samples, we hypothesized that NT may play a critical

role in MMP-9 activation. To investigate the functional role of NT signaling in gastric cancer progression, we used human gastric cancer cell lines (MKN-1 and MKN-45) and normal gastric epithelial cell lines (HFE-145) for the signaling study. We found that NT concentration was significantly higher in both cancer cell lines compared to normal gastric epithelial cell lines (Fig. 2a).

MMP-9 plays a critical role in cancer progression and invasion. We already found that MMP-9 activity was higher in the samples from gastric cancer patients. We then used gastric cancer cell lines and gastric normal epithelial cell lines for measuring the MMP-9 protein level and MMP-9 activity. MKN-1 and MKN-45 cells were treated with NT and SR48692. MMP-9 ELISA assay showed that MMP-9 secretion greatly increased in NT-treated MKN-1 and MKN-45 cells compared to HFE-145 cells, and when the cells were treated with SR48692, MMP-9 protein levels were significantly decreased (Fig. 2b, c). Further, we found that in both gastric cancer cell lines, MMP-9 activity was significantly increased when treated with NT compared with control. When the cell lines were treated with SR48692, MMP-9 activity gradually decreased. HFE-145 cell lines have lower MMP-9 activity (Fig. 2d, e). Further, we have analyzed MMP-9 activities in histological subtypes of gastric cancer plasma samples, e.g., IGC and DGC, and cell types, e.g., MKN-1 (IGC) and MKN-45

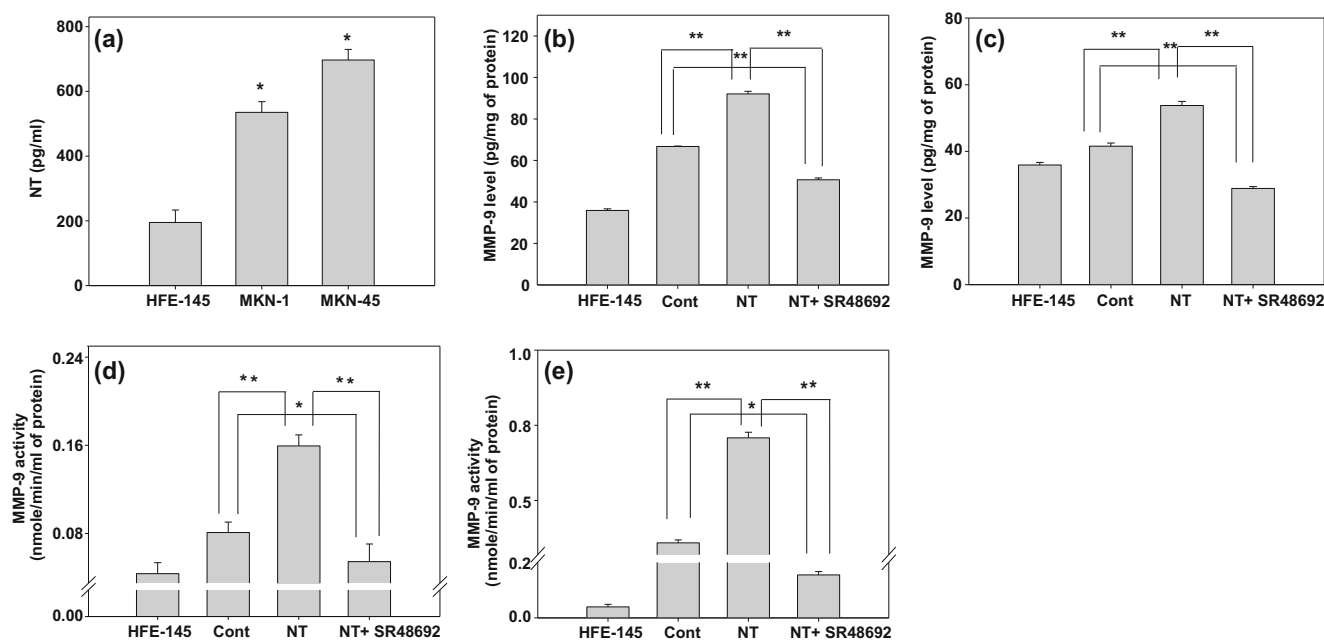


Fig. 2 NT levels and NT-mediated MMP-9 protein level as well as MMP-9 activity in normal gastric epithelial cells and gastric cancer cells. **a** MKN-1, MKN-45, and HFE-145 cells were collected and then extracted using an oasis HLB column. The extracted cells were freeze dried and later reconstituted with buffer. NT concentration was measured using NT FIA assay. The *bar* represents the standard error mean of three independent experiments conducted in triplicate. * $p < 0.05$, NT-treated MKN-1 and MKN-45 cells compared with untreated cells. Quiescent cells (b)

HFE-145, MKN-1, (c) MKN-45, and HFE-145 cells were treated with and without NT (1 μ M) for 5 min and SR48692 (10 μ M) for 30 min. Cell supernatant was collected for ELISA assay to observe the protein level of MMP-9. Quiescent cells (d) HFE-145, MKN-1, (e) HFE-145, and MKN-45 were treated with NT (1 μ M) for 5 min and SR48692 (10 μ M) for 30 min. Cells were harvested, and MMP-9 activity was measured by the fluorescence method described in the “Materials and methods” section. Results are expressed as mean \pm SEM

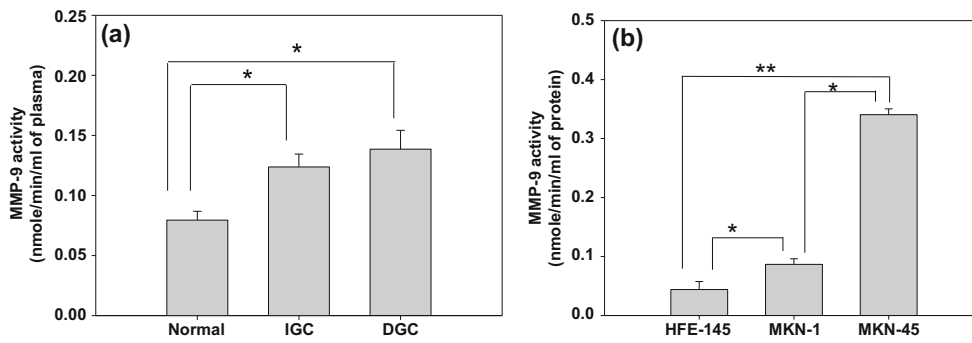


Fig. 3 MMP-9 activity was measured using fluorescence method. **a** MMP-9 activity in normal plasma, IGC, and DGC samples. **b** MMP-9 activity in HFE-145, MKN-1, and MKN-45 cell lines. Statistical

significance indicated by asterisks (*) was assessed using the GLM procedure of SAS. * $p < 0.05$, compared to normal and IGC, DGC samples. ** $p < 0.001$ compared with HFE-145 and MKN-45 cells

(DGC). Plasma MMP-9 activities in IGC and DGC were significantly higher than normal. However, there was no observed significant difference between IGC and DGC samples. As in plasma samples, MMP-9 activities were higher significantly in MKN-1 and MKN-45 cell lines compared with HEF-15 cell line. Significantly increased MMP-9 activity was also observed in MKN-45 compared to MKN-1 (Fig. 3).

MAPK signaling pathway is activated in gastric cancer cell lines by NT

MAPK and PI3K/AKT are involved in major cell signaling cascades in various human cancers. To assess the involvement of NT-stimulated effects in this pathway, we used Western blot assays to observe the activation of signaling proteins. MKN-1 and MKN-45 cells treated with 1 μ M NT experienced a rapid

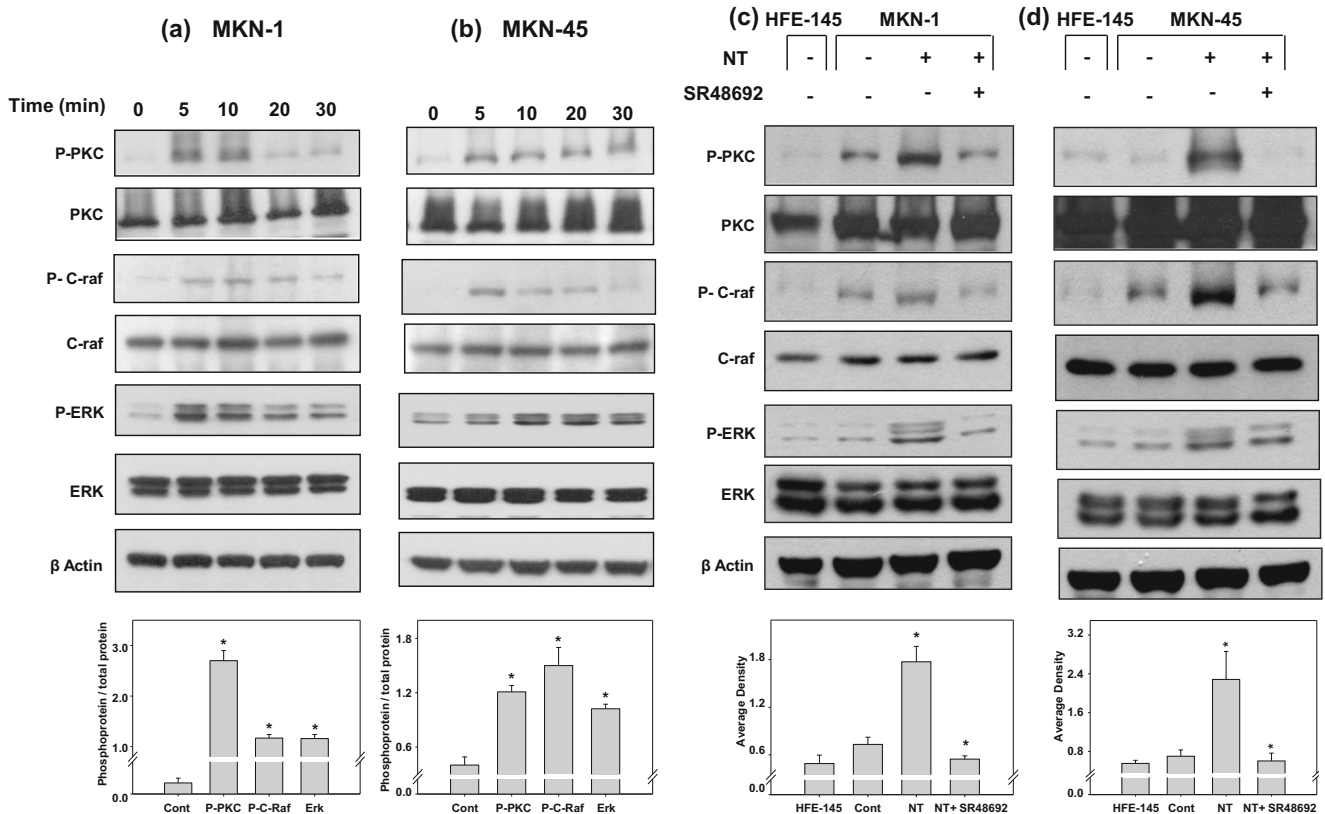


Fig. 4 NT- and SR48692-mediated phosphorylation/activation of ERK pathway cell signaling protein in gastric cancer cells. Quiescent MKN-1 (a) and MKN-45 (b) cells were incubated in the presence or absence of 1 μ M NT for 5 to 30 min. Quiescent MKN-1 (c) and MKN-45 (d) cells were pretreated with SR48692 (10 μ M) for 30 min and then stimulated with NT (1 μ M) for 10 min. The total protein was harvested for Western

blotting using phospho-specific antibodies against PKC, c-Raf, and ERK. The bar chart in lower panel shows the average densitometric analysis of the results. Results represent a typical example out of three independent experiments. Statistical significance, indicated by asterisks (*), was assessed using the GLM procedure of SAS. * $p < 0.05$ compared to control

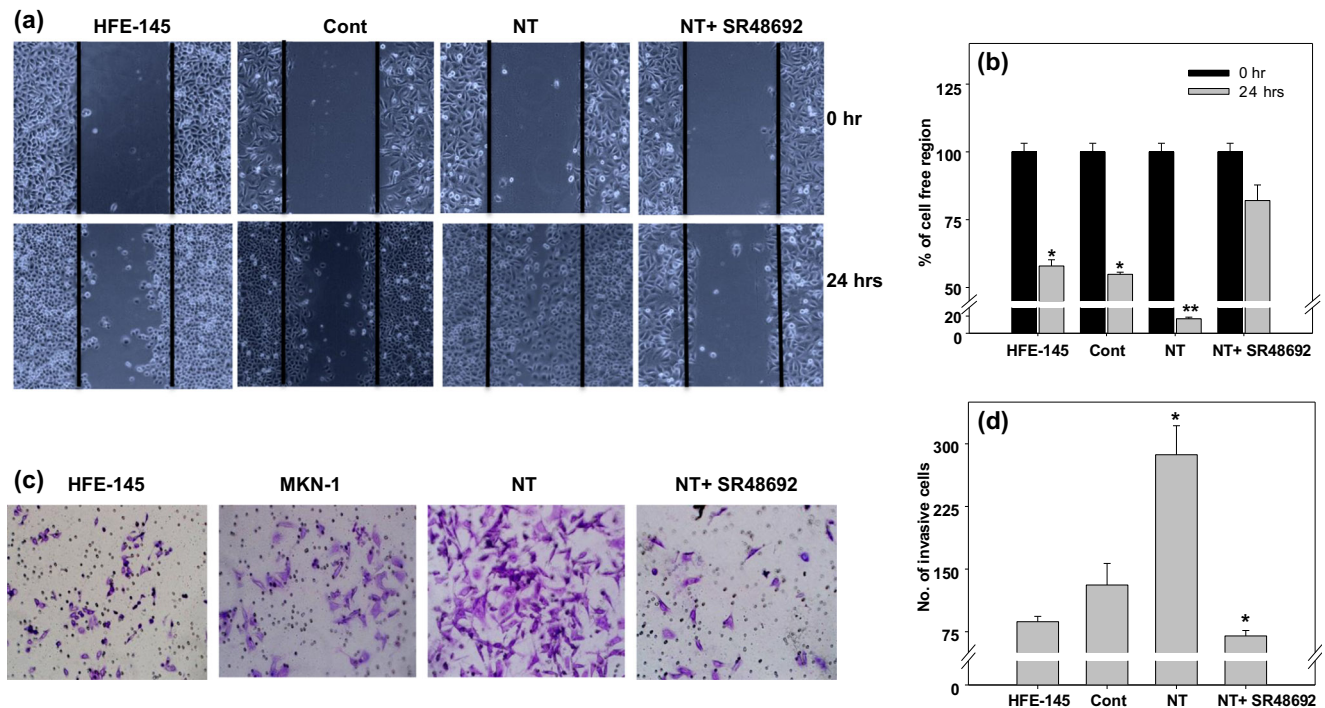


Fig. 5 Wound healing and invasion results of gastric cancer cells after NT treatment. **a** Confluent MKN-1 cell monolayers were wounded with a pipette tip and then treated with NT alone or together with SR48692. After 24 h, cell migration to the wounded area was monitored by microscopy. **b** The percentage of the total area covered by the cells and the cell free region. **c** MKN-1 cells containing serum-free media (1×10^5 cells/

well) were seeded in the upper chamber and then treated with NT alone or together with SR48692. After 24 h of incubation, cells that invaded to the surface of the insert were stained with Giemsa stain and counted using microscopy. The bars represent the standard error mean of three independent experiments. Results are expressed as mean \pm SEM. * $p < 0.05$, ** $p < 0.001$ compared with NT plus control (DMSO)-treated cells

phosphorylation of PKC and c-Raf after 5 min, which declined without a change in the total amount of PKC or c-Raf (Fig. 4a, b).

Erk is an important kinase for cell survival and metastases through the downstream activation of c-Raf [40]. Treatment with NT in gastric cancer cells increased the phosphorylation of Erk after 5 min in MKN-1 cells and after 10 min in MKN-45 cells. The phosphorylation declined without altering the total Erk (Fig. 4a, b).

Gastrointestinal hormone NT also acts as an epidermal growth factor receptor (EGFR) transactivator [41]. PKC is directly involved in the mediation of EGF receptor transmodulation [42]. In MKN-1 cells, stimulation of NT increased the phosphorylation of PI3K and Akt (Supplementary Fig. 1). These results indicated that NT caused the transactivation of EGFR via PKC, which may have secondarily activated/phosphorylated PI3K and Akt. In contrast, interestingly, NT did not affect the phosphorylation of PI3K and Akt, although we used the same concentration of NT and at the same times in MKN-45 cells (Supplementary Fig. 1).

The expression of MMP-9 appears to be highly regulated by MAPKs and NF- κ B in a variety of cell types. We found that stimulation of both cell lines with NT led to significant increases in the phosphorylation of PKC, c-Raf, and Erk compared to DMSO-treated cells. However, the expression of total

PKC, total c-Raf, and total Erk did not change in the presence of NT. To further confirm the effect of NT on the expression of MAPK activation, we inhibited the NT pathway by treating

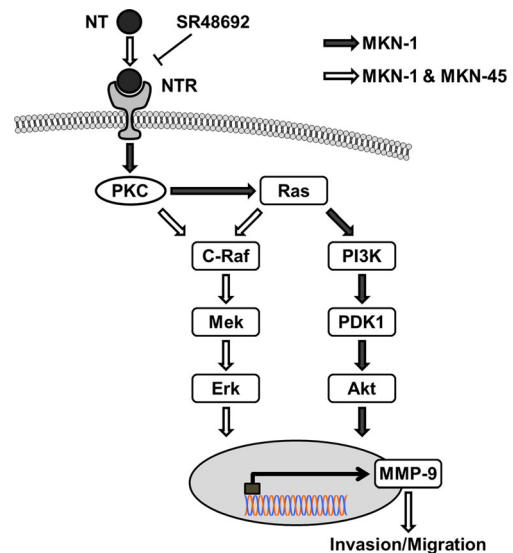


Fig. 6 Proposed signaling pathway of NT-mediated MMP-9 activation. NT induced MMP-9 activity, whereas invasion and migration are mediated through activation of PKC, c-Raf, and ERK. However, the antagonist of NTR inhibits the effects of NT by inhibiting signaling proteins

cells with SR48692 then performed Western blot analysis for phosphorylated protein. As expected, phosphorylation of PKC, c-Raf, and Erk was markedly reduced in both cells that were treated with SR48692 when compared with cells treated with control vehicle as well as HFE-145 (Fig. 4c, d), suggesting that NT signaling plays a critical role in MAPK activation.

NT enhancing the motility and invasion of gastric cancer cells

To study the effects of NT-mediated cell motility and invasion, we treated MKN-1 gastric cancer cells with NT and NT inhibitor SR48692. The MKN-1 cells were treated with NT for 24 h, whereas untreated MKN-1 and HFE-145 cells are considered as control. After 24 h, the percentages of total area covered by cell migration were measured. A significantly higher percentage of total area covered by the MKN-1 cells was observed compared to untreated control and HFE-145 cells. For further confirmation of the specificity of NT-mediated cell migration, we blocked NT activity by incubating cells with SR48692 30 min prior to NT addition. Cell migration in the inhibitor treated group was reduced compared to the NT-treated group (Fig. 5a, b).

Next, the effect of NT on the invasion of MKN-1 cells was measured using a Matrigel invasion assay. We found similar results as previously observed for cell migration; the number of cells that invaded from the upper to the lower chamber through the Matrigel was markedly higher when the cells were treated with NT. In addition, the invasive cells were fewer upon addition of NT combined with SR48692 in comparison to only NT or DMSO-treated cells (Fig. 5c, d). According to these two findings, the NT signaling pathway regulates the migration and invasion of gastric cancer cells through the activation of MMP-9.

An increase in plasma NT was observed in Korean gastric cancer patients compared to controls. The correlation between plasma NT and MMP-9 provides insight into NT-mediated invasion and metastasis in cancer cells. We confirmed the signaling mechanism of NT-mediated MMP-9 activation via MAPK/ERK pathways. The activity of NT-mediated MMP-9 activation via MAPK/ERK pathway was validated by using the specific NTR1 antagonist SR48692 (Fig. 6). Thus, our results confirmed previous results regarding increased NTR/NTR expression and induced MMP-9 expression in different cancers. We proved the relationship and underlying signaling mechanism of NT-mediated MMP-9 activation in gastric cancer.

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Conflicts of interest None

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