p75 Neurotrophin Receptor Inhibits Invasion and Metastasis of Gastric Cancer

Haifeng Jin,1,2 Yanglin Pan,1 Lijie He,1 Huihong Zhai,1 Xiaohua Li,1 Lina Zhao,1 Li Sun,1 Jie Liu,1 Liu Hong,1 Jiugang Song,1 Huahong Xie,1 Juan Gao,1 Shuang Han,1 Ying Li,1 and Daiming Fan1

1State Key Laboratory of Cancer Biology and Institute of Digestive Diseases, Xijing Hospital, the Fourth Military Medical University, Xi’an, Shaanxi Province, P.R. China and 2Department of Gastroenterology, Bethune International Peace Hospital, Shijiazhuang, Hebei Province, P.R. China

Abstract
The p75 neurotrophin receptor (p75NTR) is a focus for study at present. However, its function in gastric cancer was not elucidated. Here, we investigated its relation with metastasis of gastric cancer. By immunohistochemistry, we found that the positive rate of p75NTR expression in metastatic gastric cancer was 15.09% (16 of 106), which was lower compared with nonmetastatic gastric cancer (64.15%; 68 of 106). The average staining score in nonmetastatic gastric cancer was significantly higher than in metastatic gastric cancer (1.21 ± 0.35 versus 0.23 ± 0.18; P < 0.01). p75NTR protein level was also lowly expressed in the highly liver-metastatic gastric cancer cell line XGC9811-L compared with other gastric cancer cell lines by Western blotting. It could also significantly inhibit the in vitro adhesive, invasive, and migratory and in vivo metastatic abilities of gastric cancer cell lines SGC7901 and MKN45 by reducing urokinase-type plasminogen activator (uPA) and matrix metalloproteinase (MMP)-9 proteins and by increasing tissue inhibitor of matrix metalloproteinase (TIMP)-1 protein. Further studies showed that p75NTR could suppress the nuclear factor-κB (NF-κB) signal. SN50, a specific inhibitor of NF-κB, which could inhibit in vitro invasive and migratory abilities of gastric cancer cells, reduced expression of uPA and MMP9 proteins and increased expression of TIMP1 protein. Taken together, p75NTR had the function of inhibiting the invasive and metastatic abilities of gastric cancer cells, which was mediated, at least partially, by down-regulation of uPA and MMP9 proteins and up-regulation of TIMP1 protein via the NF-κB signal transduction pathway.

Our studies suggested that p75NTR may be used as a new potential therapeutic target in metastatic gastric cancer. (Mol Cancer Res 2007;5(5):423–33)

Introduction
Human p75 neurotrophin receptor (p75NTR), which was known as nerve growth factor receptor, maps to 17q21 (1). The gene encodes a 75-kDa cell-surface receptor glycoprotein that binds with the neurotrophin family of growth factors (2). Significantly, the p75NTR is a member of the tumor necrosis factor (TNF) receptor superfamily (3). It is now apparent that p75NTR is widely expressed in many kinds of organs, tissues, and human cancers, apart from the nervous system (2). Recent studies have shown that p75NTR has been identified as a potential tumor suppressor associated with growth inhibition, which could negatively regulate cell growth and proliferation in bladder and prostate cancer (4), and also acted as a survival receptor in brain-metastatic melanoma cells (5). Thus, it is difficult to analyze the function of p75NTR because the main physiologic role of p75NTR changes dramatically according to cell context. The aim of the present study is to know whether p75NTR is present in gastric cancer; whether its presence is related to malignant phenotype of gastric cancer; and what is the possible role of p75NTR in the invasiveness and metastasis of gastric cancer. Here, we present the first evidence that p75NTR is significantly highly expressed in nonmetastatic gastric cancers compared with metastatic gastric cancers, and we also show that p75NTR could inhibit invasion and metastasis of gastric cancer in vitro and in vivo by regulating urokinase-type plasminogen activator (uPA), matrix metalloproteinase (MMP)-9, and tissue inhibitor of matrix metalloproteinase (TIMP)-1 proteins, at least partially, via the nuclear factor κB (NF-κB) signal transduction pathway.

Results
p75NTR was Overexpressed in Nonmetastatic Gastric Cancer
We investigated the relationship between the expression of p75NTR and metastasis of gastric cancer. We first compared the expression of p75NTR in primary sites with corresponding metastatic sites of lymph node from 106 patients with metastatic gastric cancers (Table 1). It was found that p75NTR was predominantly located in the cytoplasm and membrane of gastric cancer cells (Fig. 1A). The average
staining scores of primary sites and corresponding metastatic sites of lymph node were $0.23 \pm 0.18$ and $0.19 \pm 0.16$, respectively (Fig. 1B). No significant difference in intensity of immunoreactivity or average staining score of p75NTR was found between the primary and metastatic sites from the same patients (data not shown). We then compared expression of p75NTR in primary sites from 106 patients enduring nonmetastatic gastric cancers with those from 106 patients enduring metastatic gastric cancers (Table 1). The results showed that the positive rate of p75NTR expression in metastatic gastric cancer was 15.09% (16 of 106), which was lower compared with nonmetastatic gastric cancer (64.15%; 68 of 106). According to the staining scores, the average staining score in nonmetastatic gastric cancer was significantly higher than that in metastatic gastric cancer ($1.21 \pm 0.35$ versus $0.23 \pm 0.18; P < 0.01$). These results suggested that the expression of p75NTR could not be used to discriminate primary site from metastatic site of the same metastatic gastric cancer. We also used Western blotting to detect the expression of p75NTR protein in gastric cancer cell lines (Fig. 1C). Our data confirmed that the expression of MMP9, uPA (6, 8), which had been shown to have the potential to inhibit metastasis of gastric cancer.

p75NTR Inhibited the Adhesive, Invasive, Migratory, and In vivo Metastatic Abilities of Gastric Cancer Cells

The gastric cancer cell lines SGC7901 and MKN45 were chosen for further cellular assay. pcDNA-p75NTR could up-regulate the expressions of p75NTR in SGC7901 and MKN45. The expressions of p75NTR were similar in SGC7901 and MKN45 cells transfected with SGC7901- and MKN45-pSilencer and SGC7901- and MKN45-pcDNA.

To evaluate the effect of p75NTR on cell adhesion, the ability of SGC7901- and MKN45-p75NTRS1I to adhere to Matrigel (a solubilized basement membrane rich in extracellular matrix) was investigated by adhesive assay. The expression of p75NTR was similar in SGC7901 and MKN45 cells transfected with SGC7901- and MKN45-pSilencer and SGC7901- and MKN45-pcDNA.

Table 1. The Expression of p75NTR in Primary Sites and Metastatic Sites of Metastatic Gastric Cancer and in Nonmetastatic Gastric Cancer

<table>
<thead>
<tr>
<th>Cases</th>
<th>–</th>
<th>+</th>
<th>++</th>
<th>+++</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary site of metastatic gastric cancer</td>
<td>106</td>
<td>90</td>
<td>9</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Metastatic site</td>
<td>106</td>
<td>91</td>
<td>11</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Nonmetastatic gastric cancer</td>
<td>106</td>
<td>38</td>
<td>23</td>
<td>35</td>
<td>10</td>
</tr>
</tbody>
</table>

NOTE: Interpretation of p75NTR staining was described in Materials and Methods. p75NTR staining was graded as negative (–; score: 0–0.33), weak (+; 0.34–0.99), moderate (++; 1–1.99), and strong (+++; 2–3).

uPA, MMP9, and TIMP1 Were Involved in Invasion of Gastric Cancer Regulated by p75NTR

Extracellular matrix degradation is an essential step in tumor invasion and metastasis, which was mainly mediated by the balance between some MMPs, such as MMP2 and MMP9 (9, 10), and TIMPs, such as TIMP1 and TIMP2 (11, 12), as well as the serine protease uPA protein (13, 14). Therefore, we examined the effect of p75NTR on the expressions of uPA, MMP2, MMP9, TIMP1, and TIMP2 in gastric cancer cells after transfection. Our data confirmed that the expression of MMP9 in cytoplasm (inactive form, 92 kDa) and supernatant (active form, 86 kDa) of SGC7901 could both be down-regulated by p75NTR and up-regulated by p75NTRs1I; similar results were found with uPA. However, the result of TIMP1 was on the contrary (Fig. 4A). The expression levels of MMP2 and TIMP2 were not changed by p75NTR. The levels of the β-actin loading control between samples seemed to be similar. The
levels of uPA, MMP9, and TIMP1 proteins in p75NTR- and p75NTRsi1-transfected cells were significantly different compared with the empty vector–transfected cells, which was indicated by the ratios of uPA/actin, MMP9/actin, and TIMP1/actin, respectively. In addition, the results were similar in MKN45 cells. To further confirm the role of p75NTR in the regulation of expression of uPA, MMP9, and TIMP1, we used Δp75NTR, which functioned as the dominant negative antagonist of p75NTR, which has already been shown in in vitro assay in gastric cancer. In comparison with mock transfected cells, transfection with the Δp75NTR rescued the corresponding levels of uPA and MMP9 proteins and suppressed TIMP1 protein level (0.1 or 1 μg/mL) before carrying out invasion assay. The results showed that treatment with uPA or MMP9 antibody could inhibit the invasive ability of SGC7901 and MKN45 cell lines. Inhibiting rates caused by uPA and MMP9 antibodies at a concentration of 1 μg/mL in SGC7901-p75NTRsi1 were 51.3% and 45.4%, respectively, which were both significantly higher than in SGC7901-p75NTR cells (19%), and in MKN45-p75NTRsi1 were 45.8% and 52.6%, respectively, which were both significantly higher than in MKN45-p75NTR cells (14%; Fig. 4C and D). Taken together, these results suggested that the inhibiting effect of p75NTR on metastasis of gastric cancer was at least partially mediated by down-regulation of uPA and MMP9 and by overexpression of TIMP1 protein, which possibly caused the consequent degradation of extracellular matrix.

**NF-κB Signal Transduction Pathway Was Involved in p75NTR-Related Invasion and Metastasis of Gastric Cancer**

The NF-κB signaling pathway has been recognized as “cell survival and antiapoptosis signaling” through up-regulation of several genes involved in cell proliferation and cell transformation (7). By Western blot, in SGC7901 cells we found that up-regulating p75NTR could reduce the protein levels of IκB kinase (IKK)-α, IKKβ, NF-κB (p65/RelA subunit), and p-IκBα, but could increase IκBα level. Silencing it with siRNA could increase the protein levels of IKKα, IKKβ, p65/RelA subunit, and p-IκBα, but decrease IκBα level. Δp75NTR could rescue the expression levels of IKKα, IKKβ, p65/RelA subunit, and p-IκBα proteins and suppress the expression of IκBα protein. All examined gene expression levels quantitatively analyzed and expressed as the ratios over β-actin (Fig. 5A). The results were similar in MKN45 cells (data not shown). All these results indicated that p75NTR could suppress the NF-κB signal.
To investigate whether NF-κB was necessary for the inhibiting effects of p75NTR on invasion and migration of gastric cancer and for regulating the expression of uPA, MMP9, and TIMP1, we treated cells with a specific inhibitor for nuclear translocation of NF-κB, SN50, and its inactive mutated form, SN50M, which could be used as a negative control. The results showed that total RelA (65 kDa) and phospho-RelA (85 kDa) could be decreased by SN50 compared with the control, indicating the suppression of NF-κB signaling by SN50 (Fig. 5B). After treatment with SN50 at a concentration of 50 μg/mL, the protein expressions of uPA and MMP9 were down-regulated and the protein expression of TIMP1 was up-regulated in gastric cancer cells, whereas SN50M had no effect, indicating that the nuclear translocation of NF-κB was required for the activation of uPA, MMP9, and TIMP1 expression (Fig. 5C). SN50 could also inhibit the invasive and migratory abilities of transfected gastric cancer cells. The inhibiting rates of SN50 on invasion and migration were 48.2% and 45.9%, respectively, in p75NTRsi1, significantly higher than that of SGC7901-p75NTR cells (12.5% and 8.6%, respectively;
Fig. 5D1 and D2). These results were similar in MKN45 cells. These alterations indicated that p75NTR reduced the protein levels of IKKα and IKKβ, which inhibited the phosphorylation of IkBα, accompanied by the suppression of the active forms of NF-κB RelA subunit. Thus, the NF-κB signal transduction pathway was inhibited, which led to a reduction in the expression of uPA and MMP9 and an increase in the expression of TIMP1.

Discussion

Metastasis is a mortal factor for gastric cancer patients. By now, the exact metastatic mechanisms of gastric cancer are still not fully elucidated. In the present study, we showed that p75NTR had the ability to inhibit metastasis of gastric cancer. p75NTR is a member of the TNF receptor superfamily (3), which exerts diverse functions during neuronal development in which the mechanisms have remained elusive (10). It has been shown to mediate cell death and proliferation in many different cell types, dependent on the environment of the cell (15). Except for the nervous system (2), it is widely expressed in many kinds of organs, tissues, and carcinomas such as breast cancer (16), acute leukemia (17), papillary thyroid carcinoma (18), human pancreatic cancer (19), and carcinoma of prostate (20). In this study, we first examined its expression in gastric carcinoma tissues and gastric cancer cell lines by immunohistochemistry and Western blotting, respectively. We got the result that p75NTR was significantly lowly expressed in metastatic gastric cancers compared with nonmetastatic ones and its expression level was significantly lower in highly liver-metastatic gastric cancer cell lines than in other gastric cancer cell lines. These data suggested that p75NTR may participate in the process of invasion and metastasis and exert the function of inhibiting metastasis of gastric cancer. To confirm the extrapolation from the results of immunohistochemistry and Western blotting, we did in vitro adhesion, invasion, and migration assays and in vivo metastatic assay. Evidence showed that p75NTR could significantly inhibit invasion and metastasis of gastric cancer.

In previous studies, evidence has been accumulated that invasion and metastasis of solid tumors require the action of tumor-associated proteases, which promote the dissolution of the surrounding tumor matrix and the basement membrane (21). It has also been shown that uPA, MMPs, and their tissue inhibitors (TIMPs) played an important role in that process in gastric cancer (22-25). In this study, we observed the relation of p75NTR with them. We found that p75NTR expression was associated with reduced levels of uPA and MMP9 proteins and increased level of TIMP1. Transient transfection with the Δp75NTR rescued the corresponding levels of uPA and MMP9 and suppressed the TIMP1 level, which indicated that the p75NTR was an upstream molecule of uPA, MMP9, and TIMP1 in gastric cancer. Regulated by p75NTR, uPA and MMP9 could degrade extracellular matrix and basement membrane thereby mobilizing growth factors that may promote survival, cell migration, and invasion (26). Moreover, uPA could directly activate MMP9 (27), which further contributed to extracellular degradation during invasion and tumor progression. However, the expression of MMP2 and TIMP2 did not change, accompanied by the alteration of p75NTR, which suggested that in gastric cancer cells the expression of MMP2 and TIMP2 may not be regulated by p75NTR. They may be involved in other unidentified mechanisms thereby degrading extracellular matrix and participating in the invasion and metastasis of gastric cancer. It seems that this result agrees with the observation that MMP2 is a consistent prognostic factor in gastric cancer (28). In short, in vitro and in vivo evidence showed that p75NTR had the ability to inhibit the invasiveness and metastasis of gastric cancer by regulating the expressions of uPA, MMP9, and TIMP1.

However, through which downstream signaling intermediates could p75NTR regulate the expression of uPA, MMP9, and TIMP1 in gastric cancer? Because p75NTR shares similar sequence motifs of “death domain” with TNF receptor superfamily (29), we guess it might have a similar signal transduction pathway with TNF receptor. To elucidate the signaling mechanism, we initially examined the association of the reexpression of p75NTR protein with some of the components that mediate death domain signal transduction in the TNF receptor superfamily. Previous studies have shown that the NF-κB signal transduction pathway is an important pathway of TNF receptor family members (30). It may function as an...
effector of transcription for growth and proliferation (31, 32). Earlier studies have also shown that uPA and MMP9 could be transactivated through the NF-κB pathway (33-37). Hence, we hypothesize that p75NTR may regulate uPA, MMP9, and TIMP1, at least partially, via the NF-κB signal transduction pathway thereby exerting its function as a metastasis suppressor in gastric cancer. The most important upstream intermediates of NF-κB were IKKα and IKKβ. It has been shown that IκBα binds to NF-κB and inactivates it, and that activation of NF-κB is preceded by dissociation from IκBα via its phosphorylation and degradation (38). Activation of IKKα and IKKβ could lead to IκBα phosphorylation. In our studies, overexpression of the p75NTR protein seemed to reduce the protein levels of IKKα, IKKβ, RelA, and p-IκBα and suppress the active forms of NF-κB were IKKα and IKKβ. It has been shown that IκBα binds to NF-κB and inactivates it, and that activation of NF-κB is preceded by dissociation from IκBα via its phosphorylation and degradation (38). Activation of IKKα and IKKβ could lead to IκBα phosphorylation. In our studies, overexpression of the p75NTR protein seemed to reduce the protein levels of IKKα, IKKβ, RelA, and p-IκBα and suppress the active forms of NF-κB were IKKα and IKKβ. It has been shown that IκBα binds to NF-κB and inactivates it, and that activation of NF-κB is preceded by dissociation from IκBα via its phosphorylation and degradation (38). Activation of IKKα and IKKβ could lead to IκBα phosphorylation. In our studies, overexpression of the p75NTR protein seemed to reduce the protein levels of IKKα, IKKβ, RelA, and p-IκBα and suppress the active forms of NF-κB were IKKα and IKKβ. It has been shown that IκBα binds to NF-κB and inactivates it, and that activation of NF-κB is preceded by dissociation from IκBα via its phosphorylation and degradation (38). Activation of IKKα and IKKβ could lead to IκBα phosphorylation. In our studies, overexpression of the p75NTR protein seemed to reduce the protein levels of IKKα, IKKβ, RelA, and p-IκBα and suppress the active forms of NF-κB were IKKα and IKKβ. It has been shown that IκBα binds to NF-κB and inactivates it, and that activation of NF-κB is preceded by dissociation from IκBα via its phosphorylation and degradation (38). Activation of IKKα and IKKβ could lead to IκBα phosphorylation. In our studies, overexpression of the p75NTR protein seemed to reduce the protein levels of IKKα, IKKβ, RelA, and p-IκBα and suppress the active forms of NF-κB were IKKα and IKKβ. It has been shown that IκBα binds to NF-κB and inactivates it, and that activation of NF-κB is preceded by dissociation from IκBα via its phosphorylation and degradation (38). Activation of IKKα and IKKβ could lead to IκBα phosphorylation. In our studies, overexpression of the p75NTR protein seemed to reduce the protein levels of IKKα, IKKβ, RelA, and p-IκBα and suppress the active forms of
NF-κB RelA subunit, while increasing the levels of IκBα. Transient transfection of Δp75NTR rescued the protein levels of RelA and its upstream intermediates. These results confirmed that the observed correlation between p75NTR levels and changes in the NF-κB pathway was linked directly upstream to the activation of the p75NTR protein. To investigate the effect of p75NTR on invasion and migration of gastric cancer via the NF-κB pathway, we used a specific inhibitor for NF-κB, SN50. The results that SN50 inhibited the invasive and migratory abilities of gastric cancer cells further showed that p75NTR could inhibit metastasis of gastric cancer, at least partially, via the NF-κB signal transduction pathway. In addition, to determine whether nuclear translocation of NF-κB, which was regulated by p75NTR in gastric cancer, was required for regulation of the uPA, MMP9, and TIMP1 expressions, we treated cells with SN50 and SN50M. Furthermore, evidence that SN50 could reduce the expression levels of uPA and MMP9 while increasing the expression level of TIMP1 indicated that the nuclear translocation of NF-κB was required for activation of uPA, MMP9, and TIMP1. Here, the aim of this

**FIGURE 5.** The effect of p75NTR on uPA, MMP9, and TIMP1 via the NF-κB signal transduction pathway in SGC7901 and MKN45 gastric cancer cell lines. All examined gene expression levels were quantitatively analyzed and expressed as the ratios over β-actin. Representative of three experiments with similar results. **A.** The expression of IKKα, IKKβ (p65/RelA subunit), p-IκBα, and IκBα was evaluated before and after transient transfection with Δp75NTR by Western blot. β-Actin was used as an internal control. **B.** The expression of p-RelA and RelA was evaluated after using SN50 by Western blot. β-Actin was used as an internal control. **C.** The expression of uPA, MMP9, and TIMP1 was evaluated after SN50M and SN50 treatment by Western blot. β-Actin was used as an internal control. **D.** The effect of SN50 on invasion and migration of SGC7901 and MKN45 gastric cancer cells. *, P < 0.05; **, P < 0.01 versus control and SN50M treatment.
inhibitory experiment was to further confirm that p75NTR could exert the regulatory function on uPA and MMP9 through the NF-κB signaling pathway. Of course, other molecules could also exert the same function through the NF-κB signaling pathway. The specific inhibitor of NF-κB, SN50, inhibited the expression of uPA and MMP9, whereas p75NTR could also inhibit the NF-κB signaling pathway, which further indicated that p75NTR could inhibit the expression of uPA and MMP9. Taken together, it seemed to be reasonable that p75NTR regulated the expression of uPA, MMP9, and TIMP1 via the NF-κB signal transduction pathway thereby putting its inhibiting effect on metastasis of gastric cancer (Fig. 6). This result was consistent with that of previous studies in which it has been shown that p75NTR mediated the signal transduction cascade through the NF-κB pathway to inhibit cell survival (8).

Clearly, in short, we provide the evidence that p75NTR is highly expressed in nonmetastatic gastric cancer, and the data indicate that p75NTR can inhibit metastasis of gastric cancer, at least in part, by down-regulating uPA and MMP9 and up-regulating TIMP1 via the NF-κB signal transduction pathway.

Materials and Methods

Tissue Collection and Immunohistochemistry

Tissue samples from 106 nonmetastatic gastric cancers and 106 metastatic gastric cancers were obtained from patients who underwent surgery at the Department of General Surgery in Xijing hospital, Xi’an, China. All patients having surgical tissues dissected for the study signed informed consent. All cases of gastric cancer were clinically and pathologically confirmed (data not shown). The protocols used in the study were approved by the hospital’s Protection of Human Subjects Committee. Four-micrometer sections of formalin-fixed paraffin-embedded specimens were made. Slides were dewaxed, rehydrated, incubated in 10% normal goat serum and 0.3% Triton X-100 in phosphate buffer saline (PBS) for 1 h, and then incubated with monoclonal anti-p75NTR antibody (1:100; Sigma). The slides were washed in PBS thrice for 5 min each. The tissues were incubated in biotin-labeled rabbit anti-mouse serum (1:200) for 30 min, rinsed with PBS, and incubated with avidin-biotin-peroxidase complex for 1 h. The signal was detected with 3,3-diaminobenzidine as the chromogen. Negative control slides using anti-6His as the primary antibody were included in all assays. Result was evaluated by the following formula, as previously described (39): staining score = intensity of immunoreactivity × proportion of positively staining cells. The intensity of immunoreactivity was stratified into four categories: 0, no immunoreactivity; 1, weak immunoreactivity; 2, moderate immunoreactivity; and 3, strong immunoreactivity. The proportion of positive cells was classified into four groups: 0, 0% to 5% of tumor cells exhibiting immunoreactivity; 0.33, 5% to 33% of tumor cells exhibiting immunoreactivity; 0.67, 33% to 67% of tumor cells exhibiting immunoreactivity; and 1, 67% to 100% of tumor cells exhibiting immunoreactivity.

Cell Culture

Human gastric cancer cell lines SGC7901, AGS, MKN45, BGC823, KATOIII, and XGC9811-L (40), previously described (41), were maintained on cell plates at 37°C, 5% CO2 in DMEM (Life Technologies, Inc.) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 0.1 mg/mL streptomycin. Before in vitro and in vivo assays, gastric cancer cells were pretreated with nerve growth factor (2.5S NGF, BD Biosciences) for 24 h at concentrations of 50 ng/mL as described by others (42).

Adhesion Assay

The abilities of gastric cancer SGC7901 and MKN45 cells to adhere to Matrigel were determined in 24-well plates as described by others (43). The plate surface was covered with 0.2 mL of 50 μg/mL Matrigel, incubated for 2 h, and the supernatant was removed. A suspension (0.5 mL) of tumor cells

![FIGURE 6](https://mcr.aacrjournals.org/)

**FIGURE 6.** Schematic representation of the inverse signaling effect of p75NTR expression in gastric cancer cells. Increased levels of p75NTR result in the suppression of the NF-κB pathway causing reduction in uPA and MMP9 expressions and increase in TIMP1 expression thereby inhibiting metastasis of gastric cancer. Decreased levels of p75NTR result in up-regulation of the NF-κB pathway causing increased uPA and MMP9 and reduced TIMP1 thereby facilitating metastasis of gastric cancer.
and MKN45) were suspended at 2 × 10⁵/mL) was transferred into the covered wells. After 0.5, 1, 2, and 4 h of incubation at 37°C, the adhesive cells were washed with PBS twice and then counted under a microscope at ×200 magnification on 10 random fields in each well. Each experiment was done in triplicate.

**Invasion and Migration Assay**

Cell invasion assays were done as described by others (44) using Transwells (8-μm pore size, Corning Costar Corp.). Matrigel was diluted to a concentration of 2 mg/mL, and 50 μL of this solution were placed on the lower surface of a polycarbonate filter and air-dried. After being rinsed with PBS, the filters were placed into wells and 700 μL of DMEM containing 10% bovine serum were added into the lower chamber. After incubation, the filters were placed into wells and 700 μL of DMEM containing 10% bovine serum were added into the lower compartment. Freshly trypsinized and washed cells (SGC7901 and MKN45) were suspended at 2 × 10⁵/mL in DMEM containing 1% bovine serum and were preincubated for 10 min with or without blocking antibodies. In some cases, SNS50 was added to the upper and lower chambers at a concentration of 50 μg/mL, as described by others (45). The cell suspension (150 μL) was placed into upper compartments, and the cells were allowed to invade for 36 h at 37°C in a 5% CO₂ humidified incubator. After incubation, cells were removed from the upper surface of the filter with the cotton swab; the cells that had invaded into the bottom surface of the filter were fixed with methanol and stained with hematoxylin and eosin. The invasive ability was determined by the number of penetrating cells under a microscope at ×200 magnification on 10 random fields in each well. Each experiment was done in triplicate. The method of *in vitro* migration assay, which was analyzed as previously described (46) using Transwells (8-μm pore size, Corning Costar) without Matrigel, was similar with the invasion assay. The cells (SGC7901 and MKN45) were suspended at 2 × 10⁵/mL. The incubation time was 24 h.

**Tail Vein Metastatic Assay**

The tail vein metastatic assay was analyzed as previously described (47). Nude mice were handled using best humane practices and were cared for in accordance with NIH Animal Care and Use Committee guidelines. Cells were harvested from tissue culture flasks using trypsin and washed thrice with PBS. Mice were injected with 1 × 10⁶ cells in 0.1-ML PBS through tail vein. The mice were then monitored for overall health and total body weight. After 4 weeks of injection, the mice were sacrificed. The liver tissues were observed with naked eyes and the number of visible tumors in liver surface was counted. The liver tissues were cut into serial sections before H&E staining and observed under a light microscope. Experimental and control groups contained 6 to 10 mice.

**Plasmid Construction and Cell Transfection**

pSilencer3.1 (Ambion) was used for construction of human p75NTR siRNA vectors p75NTRsi1 and p75NTRsi2 according to manufacturer’s protocol. Two pairs of specific oligonucleotides (P1, 5'-GATCCCGACAGCTGAAGGAACAGGTTCAGAGAAGCTGGTTCTGGTTCTGTCAGCTGGCAGCTGCG-3'; P2, 5'-GATCCCGACAGCTGAAGGAACAGGTTCAGAGAAGCTGGTTCTGGTTCTGTCAGCTGGCAGCTGCG-3') were annealed and then subcloned into the BamHI/HindIII cloning sites of pSilencer3.1. Full-length human p75NTR vector (pcDNA3.1-p75NTR) and the dominant negative antagonist Δp75NTR were gifts from Prof. Barbara Hempstead (Weill Medical College of Cornell University, New York, NY) and Prof. Moses V. Chao (New York University School of Medicine, New York, NY). Cell transfection was done with Lipofectamine 2000 (Invitrogen) as described in the manufacturer’s protocol. Briefly, cells were plated and grown to 70% to 90% confluence without antibiotics and then transfected with 1-μg plasmids. For stable transfection, G418 (400 μg/mL) was added into cells after 24 h of transfection. The stably transfected cells were transiently transfected with the pcDNA3.1 plasmid and truncated p75NTR intracellular domain deleted (pcDNA3.1-Δp75NTR) DNA (8). For transient transfection, cells were harvested for further experiments after 48 h of transfection. In some cases, the stably transfected cells were pretreated with SNS50 at a concentration of 50 μg/mL for 24 h and were harvested for further experiments (45). Mixed clones were screened and expanded for an additional 6 weeks. Gastric cancer cell lines SGC7901 and MKN45 transfected with pcDNA3.1-V5/His-p75NTR B, p75NTRsi1, p75NTRsi2, pSilencer, and pcDNA3.1-V5/His B were designated as SGC7901- and MKN45-p75NTR, SGC7901- and MKN45-p75NTRsi1, SGC7901- and MKN45-p75NTRsi2, SGC7901- and MKN45-pSilencer, and SGC7901- and MKN45-pcDNA, respectively.

**Western Blot**

Cells were washed twice with Hanks balanced salt solution and lysed directly in radioimmunoprecipitation assay buffer [50 mMol/L Tris-HCl (pH 7.4), 1% (v/v) Triton X-100, 1 mmol/L EDTA, 1 mmol/L leupeptin, 1 mmol/L phenylmethylsulfonyl fluoride, 10 mmol/L NaF, 1 mmol/L Na₃VO₄]. The lysates were centrifuged at 14,000 rpm for 30 min at 4°C and the supernatants were collected. To detect the expression of secreted active form of MMPs in supernatant, 10 mL of conditioned medium were concentrated 100-fold in 10-kDa microcentrifuge concentrators (Millipore). In some cases, cells were pretreated with SNS50 at a concentration of 50 μg/mL for 24 h (45). Cell lysate (60 μg) or supernatant proteins (10 μg) were respectively separated by SDS-PAGE, blotted onto nitrocellulose membrane, and incubated with a primary antibody: mouse monoclonal anti-human p75NTR (diluted 1:300; Sigma Chemical Co.); mouse monoclonal anti-uPA (diluted 1:500; Sigma Chemical); mouse monoclonal anti-MMP2 and anti-MMP9 (diluted 1:300; Santa Cruz Biotechnology); mouse monoclonal anti-TIMP1 and anti-TIMP2 (diluted 1:300; Santa Cruz Biotechnology); monoclonal antibodies for IKKα, IKKβ, NF-κB (p65/RelA subunit), and IκBα (diluted 1:500; Cell Signaling Technology); monoclonal antibodies for p-IκBα (diluted 1:500; Santa Cruz Biotechnology); and mouse monoclonal anti-β-actin (diluted 1:5,000; Sigma Chemical). After repeated washing, the membranes were probed with secondary antibodies and visualized with chemiluminescence.
incubated with horseradish peroxidase-conjugated antiantimouse secondary antibody (Santa Cruz Biotechnology) diluted 1:2,000. The bands were visualized using the enhanced chemiluminescence system (Amersham Pharmacia Biotech).

Statistical Analysis
Each experiment was repeated at least thrice. Bands from Western blot were quantified with Quantity One software (Bio-Rad). Relative protein levels were calculated with β-actin as a reference. Numerical data are presented as the mean ± SE. The difference between means was analyzed with ANOVA and then a post hoc test. All statistical analyses were done using SPSS11.0 software. P < 0.05 was considered as statistically significant.

Acknowledgments
We thank Prof. Barbara Hempstead and Prof. Moses V. Chao for full-length human p75NTR vector (pcDNA1.1-p75NTR) and the dominant negative antagonist Ap75NTR, respectively, and Qiao Taidong for excellent guidance in the process of experiment.

References
33. Sliva D, English D, Lyons D, Lloyd FP, Jr. Protein kinase C induces motility of breast cancers by up-regulating secretion of urokinase-type plasminogen activator through activation of AP-1 and NF-κB. Biochem Biophys Res Commun 2002;290:552–7.
40. Shengjuan H, Xinning G, Daiming F, et al. Phage display selection of...


43. Thamilselvan V, Basson MD. Pressure activates colon cancer cell adhesion by inside-out focal adhesion complex and actin cytoskeletal signaling. Gastroenterology 2004;126:8 –18.


Downloaded from mcr.aacrjournals.org on September 27, 2017. © 2007 American Association for Cancer Research.
Molecular Cancer Research

p75 Neurotrophin Receptor Inhibits Invasion and Metastasis of Gastric Cancer

Haifeng Jin, Yanglin Pan, Lijie He, et al.


Updated version
Access the most recent version of this article at:
http://mcr.aacrjournals.org/content/5/5/423

Cited articles
This article cites 47 articles, 16 of which you can access for free at:
http://mcr.aacrjournals.org/content/5/5/423.full.html#ref-list-1

Citing articles
This article has been cited by 4 HighWire-hosted articles. Access the articles at:
/content/5/5/423.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.