Neoplasia • Vol. 9, No. 6, June 2007, pp. 471–478

www.neoplasia.com

p75 Neurotrophin Receptor Suppresses the Proliferation of Human Gastric Cancer Cells

Haifeng Jin[*,†,1], *Yanglin Pan*[*,1], *Lina Zhao*[*,1], *Huihong Zhai*[*], *Xiaohua Li*[*], *Li Sun*[*], *Lijie He*[*], *Yu Chen*[*], *Liu Hong*[*], *Yulei Du*[*] and *Daiming Fan*[*]

*State Key Laboratory of Cancer Biology and Institute of Digestive Diseases, Xijing Hospital, The Fourth Military Medical University, Xi'an, Shaanxi Province 710032, China; †Department of Gastroenterology, Bethune International Peace Hospital, Shijiazhuang, Hebei Province 050082, China

Abstract

Identifying an effective therapeutic target is pivotal in the treatment of gastric cancer. In this study, we investigated the expression of p75 neurotrophin receptor (p75NTR) in gastric cancer and the impact of its alteration on tumor growth. p75NTR expression was absent or significantly decreased in 212 cases of gastric cancers compared with the normal gastric mucosa ($P < .05$). Moreover, p75NTR expression was also lost or significantly decreased in various human gastric cancer cell lines. p75NTR inhibited *in vitro* growth activities and caused dramatic attenuation of tumor growth in animal models by induction of cell cycle arrest. Upregulation of p75NTR led to downregulation of cyclin A, cyclin D1, cyclin E, cyclin-dependent kinase 2, p-Rb, and PCNA, but to upregulation of Rb and p27 expressions. Conversely, downregulating p75NTR with specific siRNA yielded inverse results. The rescue of tumor cells from cell cycle progression by a death domain–deleted dominant-negative antagonist of p75NTR (Δp75NTR) showed that the death domain transduced antiproliferative activity in a ligand-independent manner and further demonstrated the inhibitive effect of p75NTR on growth in gastric cancer. Therefore, we provided evidence that p75NTR was a potential tumor suppressor and may be used as a therapeutic target for gastric cancer.

Neoplasia (2007) **9,** 471–478

Keywords: Proliferation, p75NTR, cell cycle, gastric cancer, growth.

Introduction

To date, gastric cancer remains a major public health problem throughout the world, particularly in Asia, although its incidence has declined in the West in the past decades [1]. Advances in the treatment of this disease are likely to come from a fuller understanding of its biology and behavior. The aggressive nature of gastric cancer is related to mutations of various oncogenes and tumor-suppressor genes [2–6]. Previous studies have indicated the role of several tumor-suppressor genes in tumor development and progression, including *E-cadherin/CDH1*, *TP53*, *p16* [2,5,7–10], *RUNX3* [11], and, most recently, the p75 neurotrophin receptor (*p75NTR*) gene.

Human p75NTR, which was known as a nerve growth factor receptor, maps to 17q21 [12]. The gene encodes a 75-kDa cell surface receptor glycoprotein that binds with the neurotrophin family of growth factors [13]. Significantly, p75NTR is a member of the tumor necrosis factor receptor superfamily [14], which exerts diverse functions during neuronal development, the mechanisms of which have remained elusive [15]. It has been shown to mediate cell death and proliferation in many different cell types, depending on the environment of the cell [16]. It is now apparent that p75NTR is widely expressed in many kinds of organs, tissues, and human cancers, such as breast cancer [17], acute leukemia [18], papillary thyroid carcinoma [19], human pancreatic cancer [20] and prostate carcinoma [21], and is not limited to the nervous system [13]. Recent studies have shown that p75NTR has been identified as a potential tumor suppressor in prostate cancer [22]. In addition, it has been identified as a survival receptor in brain-metastatic melanoma cells [23]. Thus, it is difficult to analyze the function of p75NTR because the main physiological role of p75NTR changes dramatically depending on cell context. The aim of the present study was to know: 1) whether p75NTR is present in gastric cancer; 2) whether its presence is related to the malignant phenotype and growth of gastric cancer; and 3) the possible role of p75NTR, which is involved in the growth of gastric cancer. In the present study, we found that p75NTR could regulate cell cycle effectors of gastric cancer. Induction of cell cycle arrest was, at least in part, responsible for the antitumor activity of p75NTR in gastric cancer. Our data indicated that p75NTR was a potential tumor suppressor of gastric cancer and may be used as a therapeutic target for gastric cancer.

Address all correspondence to: Daiming Fan, State Key Laboratory of Cancer Biology and Institute of Digestive Diseases, Xijing Hospital, The Fourth Military Medical University, 17 Changle Western Road, Xi'an, Shaanxi Province 710032, China. E-mail: fandaim@fmmu.edu.cn

[1]Haifeng Jin, Yanglin Pan, and Lina Zhao contributed equally to this work.

Received 6 February 2007; Revised 6 February 2007; Accepted 21 March 2007.

DOI 10.1593/neo.07175

Materials and Methods

Immunohistochemistry

Immunohistochemistry was performed as previously described [24]. Tissue samples from 212 gastric cancers and 106 normal gastric mucosae were obtained from patients who underwent surgery at the Department of General Surgery in Xijing Hospital (Xi'an, China). All patients who agreed to have their surgical tissues dissected for the study signed an informed consent. All cases of gastric cancer had been clinically and pathologically proven (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-buffered saline (PBS) for 1 hour, and then incubated with monoclonal anti-p75NTR antibody (1:100; Sigma, Swampscott, MA). The slides were washed in PBS thrice for 5 minutes each. The tissues were incubated in biotin-labeled rabbit anti-mouse serum (1:200) for 30 minutes, rinsed with PBS, and incubated with avidin–biotin–peroxidase complex for 1 hour. The signal was detected using 3,3-diaminobenzidine as chromogen. Negative control slides using anti-6His as the primary antibody were included in all assays. A positive reaction was indicated by a reddish brown precipitate in the nucleus and cytoplasm. All sections were examined independently by two investigators. Two independent investigators scored the sections without the knowledge of patient outcome (double-blinded). An average value of two independent scores was presented in the present study.

Expression of p75NTR was evaluated according to the ratio of positive cells per specimen and staining intensity, as described previously [24]. The ratio of positive cells per specimen was evaluated quantitatively and scored as follows: 0 = *staining of ≤ 1%*; 1 = *staining of 2% to 25%*; 2 = *staining of 26% to 50%*; 3 = *staining of 51% to 75%*; and 4 = *staining of > 75%* of the cells examined. Intensity was graded as follows: 0 = *no signal*; 1 = *weak*; 2 = *moderate*; and 3 = *strong*. A total score of 0 to 12 was finally calculated and graded as negative (*–; score: 0–1*), weak (*+; score: 2–4*), moderate (*++; score: 5–8*), and strong (*+++; score: 9–12*).

Cell Culture

Human SV40-transformed immortal gastric epithelial cell GES-1 and gastric cancer cell lines SGC7901, AGS, MKN45, MKN28, KATOIII, and XGC9811-L [25], as described previously [26], were maintained on cell plates at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (Gibco RL, Grand Island, NY) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin.

Plasmid Construction and Cell Transfection

pSilencer3.1 (Ambion, Austin, TX) was used for the construction of human p75NTRsiRNA vectors p75NTRsi1 and p75NTRsi2, according to the manufacturer's protocol. Two pairs of specific oligonucleotides (P1: 5′-GAT CCG CAG CTG CAA GCA GAA CAA GTT CAA GAG ACT TGT TCT GCT TGC AGC TGT TTT TTG GAA A-3′, P2′: 5′-AGC TTT TCC AAA AAA CAG CTG CAA GCA GAA CAA GTC TCT TGA ACT TGT TCT GCT TGC AGC TGC G-3′; P2: 5′-GAT CCG CAA GAC CTC ATA GCC AGC ATT CAA GAG ATG CTG GCT ATG AGG TCT TGT TTT TTG GAA A-3′, P1′: 5′-AGC TTT TCC AAA AAA CAA GAC CTC ATA GCC AGC ATC TCT TGA ATG CTG GCT ATG AGG TCT TGC G-3′) were annealed and then subcloned into the *Bam*HI/*Hin*dIII cloning site of pSilencer3.1, respectively. The full-length human p75NTR vector (pcDNA3.1-p75NTR) and the dominant-negative antagonist Δp75NTR were gifts of Professor Barbara Hempstead (Weill Medical College of Cornell University) and Moses V. Chao (New York University School of Medicine), respectively. Cell transfection was performed with Lipofectamine2000 (Invitrogen, Carlsbad, CA), 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 hours of transfection. Stably transfected cells were transiently transfected with the truncated p75NTR intracellular domain–deleted (pcDNA3.1-Δp75NTR) DNA. For transient transfection, cells were harvested for further experiments after 48 hours of transfection. Mixed clones were screened and expanded for an additional 6 weeks. The gastric cancer cell lines SGC7901 and MKN45 transfected with pcDNA3.1-V5/6His-p75NTR B, p75NTRsi1, p75NTRsi2, pSilencer, and pcDNA3.1-V5/6His B were designated as SGC7901 (or MKN45) p75NTR, SGC7901 (or MKN45) p75NTRsi1, SGC7901 (or MKN45) p75NTRsi2, SGC7901 (or MKN45) pSilencer, and SGC7901 (or MKN45) pcDNA, respectively.

Monolayer Growth Rate

The monolayer culture growth rate was determined, as described previously [27], by the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical Co., St. Louis, MO) into water-insoluble formazan by viable cells. Three thousand cells in 200 μl of medium were plated in 96-well plates and grown under normal conditions. Cultures were assayed at 0, 1, 2, 3, 4, and 5 days, and absorbance values were determined with as enzyme-linked immunosorbent assay reader (DASIT, Milan, Italy) at 490 nm. Growth curves from SGC7901 (and MKN45) pSilencer and SGC7901 (and MKN45) pcDNA cells were generated in parallel for comparison. Each experiment was performed in triplicate.

Soft Agar Clonogenic Assay

Soft agar clonogenic assay was determined as described previously [28]. Anchorage-independent growth as a characteristic of *in vitro* tumorigenicity was assessed by soft agar clonogenic assay. Briefly, cells were detached and plated in 0.3% agarose with a 0.5% agarose underlay (1×10^4 cells/well in six-well plates). The number of foci (> 100 μm) was counted after 17 days. Each experiment was performed in triplicate.

Tumorigenicity in Nude Mice

Tumorigenicity in nude mice was determined as described previously [28]. For tumorigenicity assays, four groups of five mice each were subcutaneously injected with stably transfected cells at a single site. Tumor onset was scored visually and by palpitation at the sight of injection by two trained laboratory staff at different times on the same day. Average tumor size was estimated by physical measurement of the excised tumor at the time of sacrifice. With the exception of mice with large tumor burdens, animals were sacrificed 4 weeks after injection. These tumors were verified by hematoxylin and eosin (H&E) staining. Blocks were available for further analysis.

Cell Cycle Analysis

Flow cytometry analysis was performed as described [29]. Cells were seeded overnight on 60-mm-diameter plates in a complete medium, placed in a serum-free medium for 48 hours to synchronize the cells, and then kept again in the complete medium. At 24 hours, cells were recovered. After washing with ice-cold PBS, cells were suspended in about 0.5 ml of 70% alcohol and kept at 4°C for 30 minutes. The suspension was filtered through a 50-μm nylon mesh, and the DNA content of stained nuclei was analyzed by a flow cytometer (EPICS XL; Coulter, Miami FL). Cell cycle was analyzed using Multicycle-DNA Cell Cycle Analyzed Software (FACScan, Becton Dickinson, San Jose, CA). The proliferous index (PI) was calculated as: $PI = (S + G_2)/(S + G_2 + G_1)$. Each experiment was performed in triplicate.

Western Blot Analysis

Protein extraction and immunoblot analyses were performed as described [30]. Cells were washed twice with Hanks balanced salt solution and lysed directly in RIPA buffer (50 mM Tris–HCl pH 7.4, 1% vol/vol Triton X-100, 1 mM EDTA, 1 mM leupeptin, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, and 1 mM Na_3VO_4). Lysates were centrifuged at 14,000 rpm for 30 minutes at 4°C, and supernatants were collected. Cell lysate (60 μg) was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, blotted onto a nitrocellulose membrane, and incubated with a primary antibody, including monoclonal antibodies against p75NTR (diluted 1:200; Sigma Chemical Co.); cyclin A, cyclin D1, cyclin E, PCNA, cyclin-dependent kinase (cdk) 2, and p27 (diluted 1:500; BD Biosciences, San Jose, CA); and Rb and phosphorylated Rb (diluted 1:500; Cell Signaling Technology, Beverly, MA). β-Actin was used as a loading control in all Western blot analyses (diluted 1:5000; Sigma Chemical Co.). After repeated washing, the membranes were incubated with horseradish peroxidase–conjugated anti-mouse secondary antibody (diluted 1:2000; Santa Cruz Biotechnology, Santa Cruz, CA). The bands were visualized using the enhanced chemiluminescence system (Amersham-Pharmacia Biotech, Beijing, China). Each experiment was performed in triplicate. All examined gene expression levels were quantitatively analyzed and expressed as ratios to β-actin.

Statistical Analysis

Statistical analysis was performed with Kruskal-Wallis rank test, and Mann-Whitney *U* test was used to calculate *P* values and to compare the differences between groups for immunohistochemistry. Assays for characterizing cell phenotype were analyzed by Student's *t* test. Statistical SPSS software package (SPSS, Inc., Chicago, IL) was used to analyze data. Differences were considered statistically different at $P < .05$.

Results

p75NTR Expression Was Decreased or Absent in Gastric Cancer

p75NTR expression was evaluated in the 106 normal mucosa and primary tumor tissues of all 212 patients by immunohistochemistry. It was found that p75NTR was predominantly located in the cytoplasm and membrane of gastric cancer cells (Figure 1). $p75NTR^+$ expression was found in normal gastric mucosae (88.53%) at a value higher than that in tumor tissues of gastric cancer (40.57%) ($P < .05$). When considering staining scores, the average staining score in normal gastric mucosae was significantly higher than that in gastric cancer (7.82 ± 2.14 *vs* 3.24 ± 1.53; $P < .01$) (Figure 2*A*). As shown in Table 1, in gastric cancer specimens, the expression of p75NTR protein in well-differentiated tumor tissues was higher than that in moderately or poorly differentiated ones ($P < .01$, respectively), indicating a correlation between p75NTR expression and the differentiation grade of gastric cancer. In addition, p75NTR expression in patients with lymph node and/or distant metastasis (Tumor–Node–Metastasis stages III and IV) was significantly lower than that in patients without metastasis (Tumor–Node–Metastasis stages I and II; $P < .01$), indicating a relationship between p75NTR expression and gastric cancer metastasis. To further confirm these observations, Western blot analysis was performed using four paired human normal gastric and tumor tissue specimens. It was clear that the tumor tissue specimens had a loss or a drastic decrease in p75NTR expression compared with normal gastric tissues (Figure 2*B*), which was consistent with the level of p75NTR protein expression determined by immunohistochemical staining. After that, we compared p75NTR expression in six gastric cancer cell lines (SGC7901, AGS, MKN45, MKN28, KATOIII, and XGC9811-L) [25] and one immortal gastric epithelial cell line. As shown in Figure 2*C*, p75NTR expression was higher in the immortal gastric epithelial cell line (GES-1) than in all gastric cancer cell lines.

p75NTR Inhibits In Vitro *Proliferation and Growth and* In Vivo *Tumorigenicity of Gastric Cancer Cells*

To downregulate the expression of p75NTR in gastric cancer cells, two p75NTR-specific siRNA vectors, namely, p75NTRsi1 and p75NTRsi2, were designed and constructed, aiming at 661 to 679 and 828 to 846 in the coding sequence of p75NTR, respectively. After cell transfection and antibiotic screening for 6 weeks, the expression of

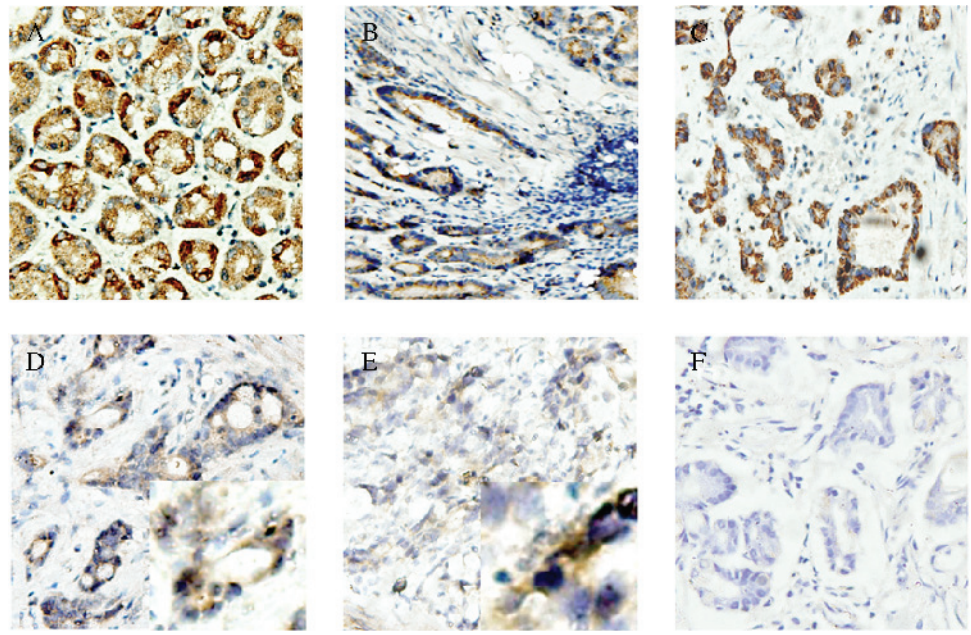

Figure 1. *Immunohistochemical staining of p75NTR in normal gastric tissues and in gastric cancer at different stages of differentiation. Monoclonal mouse anti-p75NTR antibodies were used to stain paraffin sections. (A) A normal epithelium exhibiting positive p75NTR immunostaining. (B and C) Well-differentiated, (D) moderately differentiated, and (E) poorly differentiated gastric cancer tissues showing moderate or weak p75NTR immunosignals in most epithelial cells. (F) Negative control slides using anti-6His as the primary antibody. Original magnification, ×20.*

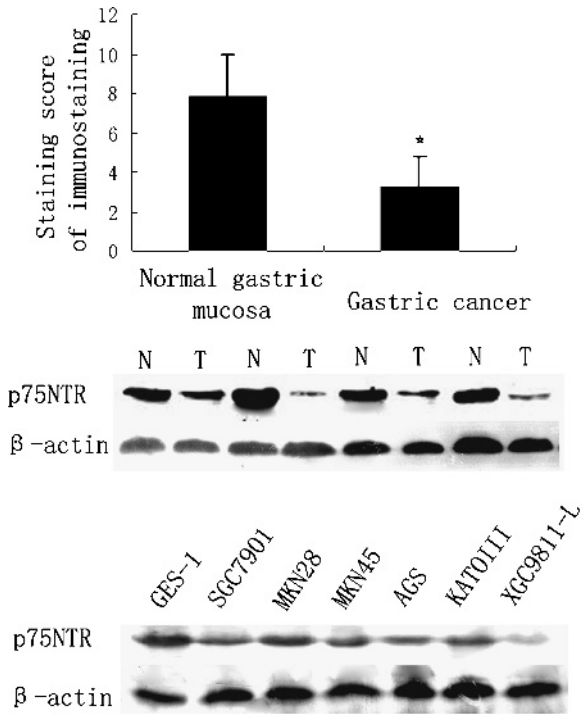

Figure 2. *Staining score of the immunostaining of p75NTR in normal gastric mucosa and in gastric cancer, and its expression in normal gastric (N) and gastric tumor tissue (T) specimens and in gastric cancer cell lines. (A) The results of immunohistochemical staining were evaluated by staining scores described in the Materials and Methods section. *P < .05 vs normal gastric mucosa. (B) Expression of p75NTR in normal gastric (N) and gastric tumor tissue (T) specimens. (C) Expression of p75NTR in gastric cancer cell lines. β-Actin was used as an internal control. Representative experiment of three, with similar results.*

p75NTR in stably transfected cells was determined by Western blot analysis. p75NTRsi1 could downregulate the expression of p75NTR in SGC7901 and MKN45 effectively, whereas the effect of p75NTRsi2 on p75NTR expression was minimal (Figure 3*A*). Then the stably transfected SGC7901-p75NTRsi1 and MKN45-p75NTRsi1 were chosen for further cellular assay. pcDNA-p75NTR could upregulate the expressions of p75NTR in SGC7901 and MKN45. The expressions of p75NTR were similar in SGC7901 and MKN45 cells transfected with empty vectors.

When the growth curves of these cell lines were compared in a medium containing 10% fetal calf serum, the curves for p75NTRsi1 cells were significantly higher than those for control cells, whereas the curves for p75NTR cells were significantly lower than those for control cells ($P < .05$ on days 4–7; Figure 3*B*). To determine the effect of p75NTR on the colony-forming ability of gastric cancer cells, we performed *in vitro* soft agar assay. The results showed that upregulating p75NTR could decrease cell growth in soft agar ($P < .05$), whereas downregulating p75NTR with siRNA could increase cell growth (Figure 3*C*). Furthermore, *in vivo* subcutaneous tumor formative assay was adopted to examine the proliferative ability of SGC7901 (and MKN45) p75NTR in nude mice. Compared with control cells transfected with empty vector, the injection of SGC7901 (and MKN45) p75NTR cells led to a significantly decreased tumor size. Conversely, inoculation of SGC7901 (and MKN45) p75NTRsi1 cells led to a significantly increased tumor size ($P < .05$; Figure 3*D*). Both *in vitro* and *in vivo* assays suggested that p75NTR had the potential to inhibit the proliferation, growth, and tumorigenicity of gastric cancer.

Table 1. Clinicopathological Associations of p75NTR Expression in Patients with Gastric Cancer.

	Total Number of Cases	p75NTR Immunostaining			
		–	+	++	+++
Normal gastric mucosa [*n* (%)]	106	12 (11.32)	7 (6.6)	24 (22.64)	63 (59.43)
Gastric carcinoma [*n* (%)]	212	128 (60.38)	30 (14.15)	42 (19.81)	12 (5.66)
Differentiation					
Well differentiated	37	9	6	12	10
Moderately differentiated	91	39	13	35	4
Poorly differentiated	84	57	21	6	0
Gross type (Borrmann)					
I (polypodi)	28	6	3	13	6
II (ulcerofungating)	39	7	13	14	5
III (ulceroinfiltrative)	113	5	32	50	26
IV (diffuse)	32	7	8	10	7
Tumor–Node–Metastasis					
I + II	66	7	19	28	12
III + IV	146	18	36	60	32
Metastasis					
With	106	84	14	6	2
Without	106	38	21	37	10

The interpretation of p75NTR staining is described in the Materials and Methods section. p75NTR staining was graded as negative (*–; score: 0–1*), weak (*+; score: 2–4*), moderate (*++; score: 5–8*), and strong (*+++; score: 9–12*).

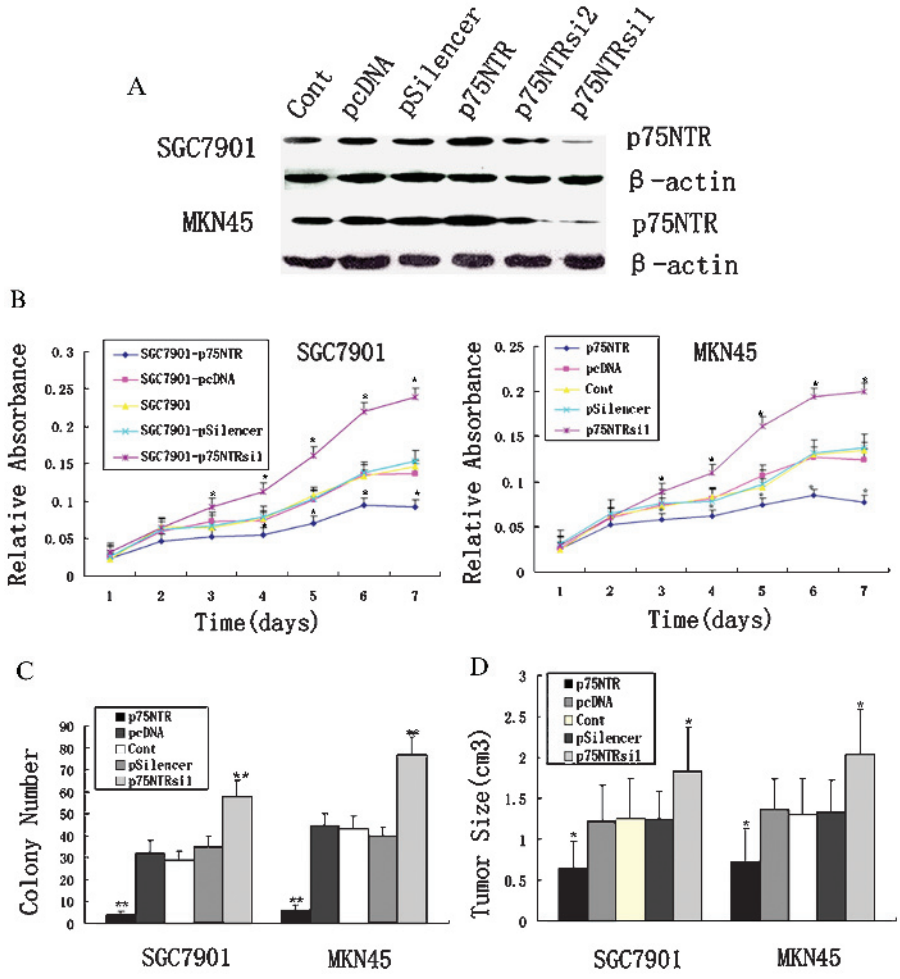

Figure 3. *Effects of p75NTR and p75NTRsiRNA on the* in vitro *proliferation and growth and* in vivo *tumorigenicity of gastric cancer cells. Representative experiment of three, with similar results. (A) After stable transfection, the expression of p75NTR was evaluated by Western blot analysis. β-Actin was used as an internal control. (B) The role of p75NTR and p75NTRsi1 in regulating the proliferation of SGC7901 and MKN45 cells. The monolayer growth rates of cells were determined by MTT assays. Values represent the mean (standard error of the mean) from at least three separate experiments. *P < .05. (C) Effect of p75NTR and p75NTRsi1 on the colony formation of SGC7901 and MKN45 cells. Cells were placed in media containing soft agar and incubated for 17 days. The number of foci (> 100 μm) was counted. Values represent the mean (standard error of the mean) from at least three separate experiments, each conducted in triplicate. **P = .000. *P < .01. (D) Effect of p75NTR and p75NTRsi1 on the tumorigenicity of SGC7901 and MKN45 cells in nude mice. Average tumor size was estimated by physical measurement of the excised tumor at the time of sacrifice. These tumors were verified as gastric cancer by H&E staining. *P < .01.*

p75NTR Induces the Cell Cycle Arrest of Gastric Cancer Cells

To further investigate the mechanism by which p75NTR inhibits gastric cancer cell growth, we studied the effects of p75NTR expression on the cell cycle by fluorescence-activated cell sorter analysis. The results of the cell cycle showed that at 24 hours after the release of synchronized cultures, 8.42% of SGC7901-p75NTR cells were in S-phase compared to 12.49% of SGC7901 cells, whereas 16.53% of SGC7901-p75NTRsi1 cells were in S-phase compared to 12.49% of SGC7901 cells ($P < .05$). The results were similar in MKN45-transfected cells. Furthermore, we used a deletion construct of p75NTR that lacks the intracellular domain (Δp75NTR) and functions as a dominant-negative antagonist of p75NTR [31,32], which had been demonstrated to have the same function in prostate cancer. We found that Δp75NTR could reverse the effect of p75NTR on the cell cycle by flow cytometry (Table 2). To further investigate the mechanism by which p75NTR induced cell cycle arrest in gastric cancer cells, we examined cell cycle effectors by Western blot analysis. Our results indicated that upregulation of p75NTR protein was associated with a reduction in cyclin A, cyclin D1, cyclin E, cdk2, p-Rb, and PCNA proteins, but with an increase in p27 and Rb proteins. However, downregulation of p75NTR protein by siRNA correlated with the increase in cyclin A, cyclin D1, cyclin E, cdk2, p-Rb, and PCNA proteins, but with reduction in p27 and Rb proteins. To further confirm the role of p75NTR in regulating cell cycle effectors, we used Δp75NTR, which functions as the dominant-negative antagonist of p75NTR. Transfection of Δp75NTR appeared to rescue the p75NTR-associated decrease in cyclin A, cyclin D1, cyclin E, cdk2, p-Rb, and PCNA, and to suppress the expression of p27 and Rb (Figure 4). The levels of β-actin loading control between samples appeared similar.

Discussion

During the carcinogenesis of stomach cancer, various genetic and epigenetic alterations accumulate to facilitate cell transformation and to enhance aggressive behaviors. Although a number of cellular genes have been identified to be involved in gastric carcinogenesis, the tumorigenesis and progression of gastric cancer have not been fully elucidated. Therefore, it is vital to identify more proteins specifically related to gastric carcinogenesis, which may expand our understanding of this disease and may develop new targets for therapy and indicators for diagnosis.

p75NTR gene is a member of the tumor necrosis factor receptor superfamily [14], which was found to be expressed in many kinds of organs, tissues, and human cancers. Its exact role in carcinomas is not fully elucidated. Above all, its expression in gastric cancer, the effect of its alterations on the growth of gastric cancer, and the use of this pathway for targeted cancer therapy have remained unelucidated. In the present study, we provided evidence that p75NTR was a tumor suppressor of gastric cancer. Specifically, we found that p75NTR protein was expressed in the membrane and cytoplasm of epithelial cells of gastric mucosa, whereas there was a significant decrease or absence of p75NTR expression in gastric cancer specimens at a high frequency. In addition, we found that $p75NTR^+$ expression in normal gastric mucosae was higher than that in tumor tissues of gastric cancer; the average staining score in normal gastric mucosae was significantly higher than that in gastric cancer. It suggested that p75NTR could play roles in gastric carcinogenesis and may have an inhibitory effect on the proliferation of gastric cancer cells. In this report, p75NTR expression was found to be downregulated in gastric cancer cell lines compared with the normal gastric epithelial cell line by Western blot analysis. Our work confirmed and extended previous observations that p75NTR is downregulated in gastric cancer.

To determine whether the ectopic expression of p75NTR could modulate the proliferation of gastric cancer cells, we introduced pcDNA-p75NTR and pSilencer-p75NTRsi1 into SGC7901 and MKN45 cells, respectively. As a result, we found that p75NTR significantly inhibited gastric cancer cell proliferation and growth *in vitro* and tumorigenicity in animal models. The stimulatory effects of pSilencer-p75NTRsi1 and the suppressive effect of pcDNA-p75NTR on SGC7901 and MKN45 cells showed that *p75NTR* gene may be a growth arrest gene that acts directly or indirectly to control the proliferation of cells. The products of such genes regulate cell growth and differentiation in a negative fashion, thus suppressing neoplastic development, which further indicated that p75NTR could act as a tumor suppressor in gastric cancer. Then we demonstrated that a component of p75NTR tumor-suppressor activity is mediated by the inhibition of cell

Table 2. Cell Cycle Analysis of p75NTR Expression in SGC7901 and MKN45 Cells.

SGC7901 (MKN45) Clones Expressing the p75NTR Protein	(A) Mock			(B) Δp75NTR		
	G_1	G_2	S	G_1	G_2	S
p75NTR	80.34 (82.13)	11.24 (10.37)	8.42 (7.5)	58.29 (61.75)	22.74 (21.36)	19.97 (16.89)
pcDNA	75.17 (74.25)	11.89 (11.86)	12.94 (13.89)	63.43 (64.37)	20.79 (22.12)	15.78 (13.51)
Cont	75.78 (74.15)	11.73 (10.78)	12.49 (15.07)	64.51 (65.48)	21.38 (21.62)	14.11 (12.9)
pSilencer	76.06 (74.95)	12.03 (11.14)	11.91 (13.91)	63.54 (65.17)	21.49 (21.91)	14.97 (12.92)
p75NTRsi1	71.89 (68.54)	11.58 (12.06)	16.53 (19.4)	69.83 (70.23)	19.74 (22.15)	10.43 (7.62)
Statistical significance	*	NS	*	*	NS	*

Enumeration (%) of cell cycle analysis in SGC7901(MKN45) clones. (A) Mock cells. (B) Death domain–deleted p75NTR-transfected cells.
NS, not significant.
*$P < .05$ (representative experiment of three, with similar results).

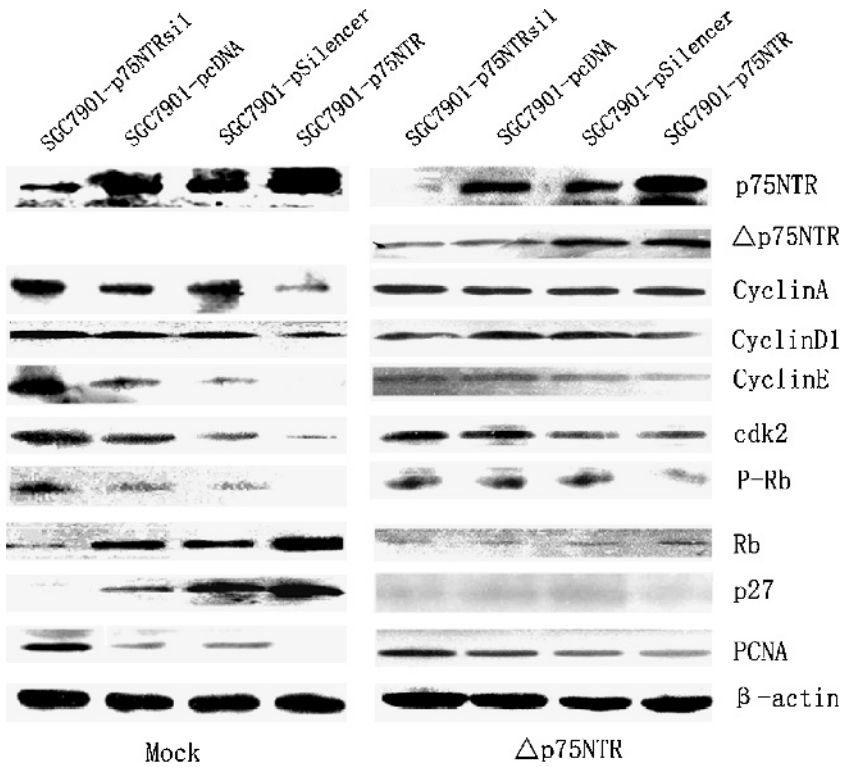

Figure 4. *The effect of p75NTR on cell cycle effectors by Western blot analysis. All examined gene expression levels were quantitatively analyzed and expressed as ratios to β-actin. Representative experiment of three, with similar results. The expressions of cyclin A, cyclin D1, cyclin E, cdk2, p-Rb, Rb, p27, and PCNA proteins were evaluated before and after transient transfection with Δp75NTR by Western blot analysis. β-Actin was used as an internal control.*

cycle progression commensurate with increased accumulation of cancer cells in G_1-phase and a corresponding reduction of cells in the S-phase of the cell cycle in the SGC7901 and MKN45 gastric cancer cell lines. Significantly, the rescue of tumor cells from retarded cell cycle progression by a death domain–deleted dominant-negative antagonist of p75NTR shows that the death domain transduced antiproliferative activity in a ligand-independent manner. The interaction of the truncated p75NTR lacking the death domain with full-length p75NTR antagonizes wild-type p75NTR protein function [32].

It is well known that cell cycle initiation and progression are regulated by several classic cdks. The expression of cyclin D complexed with PCNA has been shown to promote the phosphorylation of Rb during progression from early to mid-G_1 [33]. Beyond mid-G_1, near the end of the G_1/S restriction point, the expression of cyclin E complexed with cdk2 has been shown to promote the phosphorylation of Rb [34,35]. Beyond the G_1/S restriction point, expression of the cyclin A/cdk2 complex has been shown to maintain the phosphorylation of Rb during the S-phase of the cell cycle [36,37]. Inversely, p27 belongs to the Cip/Kip family, which acts as a broad specific inhibitor of cyclin D, cyclin E, and cyclin A complexes. The deregulation of these CDKIs is a common feature in tumor cells and mainly contributes to the disruption of cell cycle control. p27Kip1 (p27) is a CDKI that exerts its inhibitory activity on many steps of the cell cycle [38]. Clearly in our studies, p75NTR downregulated cyclin D1, PCNA, and phosphorylated Rb proteins, which indicates that p75NTR was associated with retarded progression through early to mid-G_1. In addition, the p75NTR downregulation of cyclin E and cdk2 proteins in gastric cancer cells indicates that progression through the G_1/S restriction point is regulated by p75NTR protein expression. Likewise, suppression of cdk2 and cyclin A supports the selective effect of p75NTR expression on retarding progression through the S-phase of the cell cycle, although p75NTR upregulation of p27 protein further indicated its inhibition of the cell cycle in gastric cancer. The rescue of cyclin D1, PCNA, and phosphorylated Rb levels by the death domain–deleted dominant-negative antagonist of p75NTR shows a p75NTR-dependent regulation of the cell cycle in gastric cells. Taken together, it seems clear that p75NTR expression selectively alters specific cell cycle–regulatory molecules that retard progression from early to mid-G_1, the G_1/S restriction point, and the S-phase of the cell cycle in gastric cancer.

In conclusion, p75NTR could be an effective gene therapeutic target for gastric cancer. As shown in this research, enforced p75NTR expression significantly inhibited gastric cancer growth *in vitro* and *in vivo* by induced accumulation and concomitant reduction of cells in the G_1 and S phases, respectively, of the cell cycle. Further research may help design an effective therapeutic modality to control gastric cancer.

Acknowledgements

We are grateful to Barbara Hempstead and Moses V. Chao for the full-length human p75NTR vector (pcDNA3.1-

p75NTR) and the dominant-negative antagonist Δp75NTR. We are also grateful to Taidong Qiao for excellent guidance during the experiment.

References

[1] Parkin DM, Pisani P, and Ferlay J (1999). Global cancer statistics. *CA Cancer J Clin* **49**, 33–64.

[2] Ushijima T and Sasako M (2004). Focus on gastric cancer. *Cancer Cell* **5**, 121–125.

[3] Tahara E (2000). Molecular aspects of invasion and metastasis of stomach cancer. *Verh Dtsch Ges Pathol* **84**, 43–49.

[4] Chan AO, Luk JM, Hui WM, and Lam SK (1999). Molecular biology of gastric carcinoma: from laboratory to bedside. *J Gastroenterol Hepatol* **14**, 1150–1160.

[5] Fenoglio-Preiser CM, Wang J, Stemmermann GN, and Noffsinger A (2003). *TP53* and gastric carcinoma: a review. *Hum Mutat* **21**, 258–270.

[6] Sud R, Wells D, Talbot IC, and Delhanty JD (2001). Genetic alterations in gastric cancers from British patients. *Cancer Genet Cytogenet* **126**, 111–119.

[7] Carneiro F, Huntsman DG, Smyrk TC, Owen DA, Seruca R, Pharoah P, Caldas C, and Sobrinho-Simoes M (2004). Model of the early development of diffuse gastric cancer in E-cadherin mutation carriers and its implications for patient screening. *J Pathol* **203**, 681–687.

[8] Guilford P, Hopkins J, Harraway J, McLeod M, McLeod N, Harawira P, Taite H, Scoular R, Miller A, and Reeve AE (1998). E-cadherin germline mutations in familial gastric cancer. *Nature* **392**, 402–404.

[9] El-Rifai W and Powell SM (2002). Molecular biology of gastric cancer. *Semin Radiat Oncol* **12**, 128–140.

[10] Migliavacca M, Ottini L, Bazan V, Agnese V, Corsale S, Macaluso M, Lupi R, Dardanoni G, Valerio MR, Pantuso G, et al. (2004). *TP53* in gastric cancer: mutations in the I3 loop and LSH motif DNA-binding domains of *TP53* predict poor outcome. *J Cell Physiol* **200**, 476–485.

[11] Daoyan W, Weida G, Sang C, Qiang L, Won DK, Liwei W, Xiangdong L, James Y, Tsung TW, Suyun H, et al. (2005). Loss of *RUNX3* expression significantly affects the clinical outcome of gastric cancer patients and its restoration causes drastic suppression of tumor growth and metastasis. *Cancer Res* **65**, 11.

[12] Huebner K, Isobe M, Chao M, Bothwell M, Ross AH, Finan J, Hoxie JA, Sehgal A, Buck CR, Lanahan A, et al. (1986). The nerve growth factor receptor gene is at human chromosome region 17q12–17q22, distal to the chromosome 17 breakpoint in acute leukemias. *Proc Natl Acad Sci USA* **83**, 1403–1407.

[13] Rabizadeh S and Bredesen DE (2003). Ten years on: mediation of cell death by the common neurotrophin receptor p75(NTR). *Cytokine Growth Factor Rev* **14**, 225–239.

[14] Liepinsh E, Ilag LL, Otting G, and Ibanez CF (1997). NMR structure of the death domain of the p75 neurotrophin receptor. *EMBO J* **16**, 4999–5005.

[15] Rabizadeh S, Oh J, Zhong LT, Yang J, Bitler CM, Butcher LL, and Bredesen DE (1993). Induction of apoptosis by the low-affinity NGF receptor. *Science* **261**, 345–348.

[16] Roux PP and Barker PA (2002). Neurotrophin signaling through the p75 neurotrophin receptor. *Prog Neurobiol* **67**, 203–233.

[17] Reis-Filho JS, Steele D, Di Palma S, Jones RL, Savage K, James M, Milanezi F, Schmitt FC, and Ashworth A (2006). Distribution and significance of nerve growth factor receptor (NGFR/p75NTR) in normal, benign and malignant breast tissue. *Mod Pathol* **19**, 307–319.

[18] Beutel G, Meyer J, Ma L, Yin S, Eder M, von Neuhoff N, Wilkens L, Wei J, Hertenstein B, Heil G, et al. (2005). Expression of the p75 neurotrophin receptor in acute leukaemia. *Br J Haematol* **131**, 67–70.

[19] Rocha AS, Risberg B, Magalhaes J, Trovisco V, de Castro IV, Lazarovici P, Soares P, Davidson B, and Sobrinho-Simoes M (2006). The p75 neurotrophin receptor is widely expressed in conventional papillary thyroid carcinoma. *Hum Pathol* **37**, 562–568.

[20] Dang C, Zhang Y, Ma Q, and Shimahara Y (2006). Expression of nerve growth factor receptors is correlated with progression and prognosis of human pancreatic cancer. *J Gastroenterol Hepatol* **21**, 850–858.

[21] Pflug BR, Onoda M, and Lynch JH (1992). Reduced expression of the low affinity nerve growth factor receptor in benign and malignant human prostate tissue and loss of expression in four human metastatic prostate tumor cell lines. *Cancer Res* **52**, 5403–5406.

[22] Khwaja F, Allen J, Lynch J, Andrews P, and Djakiew D (2004). Ibuprofen inhibits survival of bladder cancer cells by induced expression of the p75NTR tumor suppressor protein. *Cancer Res* **64**, 6207–6213.

[23] Dario M, Rebecca A, and Jason B (2004). p75 neurotrophin receptor functions as a survival receptor in brain-metastatic melanoma cells. *J Cell Biochem* **91**, 206–215.

[24] Maaser K, Daubler P, Barthel B, Heine B, von Lampe B, Stein H, Hoffmeister B, Scherer H, and Scherubl H (2003). Oesophageal squamous cell neoplasia in head and neck cancer patients: upregulation of COX-2 during carcinogenesis. *Br J Cancer* **88**, 1217–1222.

[25] Hu S, Guo X, Xie H, Du Y, Pan Y, Shi Y, Wang J, Hong L, Han S, Fan D, et al. (2006). Phage display selection of peptides that inhibit metastasis ability of gastric cancer cells with high liver-metastatic potential. *Biochem Biophys Res Commun* **341**, 964–972.

[26] Pan Y, Bi F, Liu N, Xue Y, Yao X, Zheng Y, and Fan D (2004). Expression of seven main Rho family members in gastric carcinoma. *Biochem Biophys Res Commun* **315**, 686–691.

[27] van Golen KL, Wu ZF, Qiao XT, Bao LW, and Merajver SD (2000). RhoC GTPase, a novel transforming oncogene for human mammary epithelial cells that partially recapitulates the inflammatory breast cancer phenotype. *Cancer Res* **60**, 5832–5838.

[28] Na L, Feng B, Yanglin P, Lijun S, Yan X, Yongquan S, Xuebiao Y, Yi Z, and Daiming F (2004). Reversal of the malignant phenotype of gastric cancer cells by inhibition of RhoA expression and activity. *Clin Cancer Res* **10**, 6239–6247.

[29] Milner AE, Levens JM, and Gregory CD (1998). Flow cytometric methods of analyzing apoptotic cells. *Methods Mol Biol* **80**, 347–354.

[30] Pan Y, Zhao L, Liang J, Liu J, Shi Y, Liu N, Zhang G, Jin H, Gao J, Fan D, et al. (2006). Cellular prion protein promotes invasion and metastasis of gastric cancer. *FASEB J* **20**, 1886–1888.

[31] Allen J, Khwaja F, Byers S, and Djakiew D (2005). The p75NTR mediates a bifurcated signal transduction cascade through the NFkappaB and JNK pathways to inhibit cell. *Exp Cell Res* **304**, 69–80.

[32] Wang X, Bauer JH, Li Y, Shao Z, Zetoune FS, Cattaneo E, and Vincenz C (2001). Characterization of a p75NTR apoptotic signaling pathway using a novel cellular model. *J Biol Chem* **276**, 33812–33820.

[33] Satyanarayana A and Rudolph KL (2004). *p16* and ARF: activation of teenage proteins in old age. *J Clin Invest* **114**, 1237–1240.

[34] Perez M, Regan T, Pflug B, Lynch J, and Djakiew D (1997). Loss of low-affinity nerve growth factor receptor during malignant transformation of the human prostate. *Prostate* **30**, 274–279.

[35] Geldof AA, Van Haarst EP, and Newling DW (1998). Neurotrophic factors in prostate and prostatic cancer. *Prostate Cancer Prostatic Dis* **1**, 236–241.

[36] Miller FD and Kaplan DR (2001). Neurotrophin signalling pathways regulating neuronal apoptosis. *Cell Mol Life Sci* **58**, 1045–1053.

[37] Fatima K, Arshia T, Jeff A, and Djakiew D (2006). The p75NTR tumor suppressor induces cell cycle arrest facilitating caspase mediated apoptosis in prostate tumor cells. *Biochem Biophys Res Commun* **341**, 1184–1192.

[38] Sherr CJ and Roberts JM (1999). CDK inhibitors: positive and negative regulators of G_1-phase progression. *Genes Dev* **13**, 1501–1512.