The Biological Role of the Low-Affinity p75 Neurotrophin Receptor in Esophageal Squamous Cell Carcinoma

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Abstract In this study, we investigated the clinicopathologic significance of the low-affinity p75 neurotrophin receptor (p75NTR; which is expressed in the stem/progenitor cell fraction of normal esophageal epithelial cells) in 187 resected esophageal squamous cell carcinoma (ESCC) specimens and found that ~50% of ESCC expressed p75NTR. Our investigation using ESCC cell lines showed that p75NTR was intensely expressed in the cells with high colony-forming capacity but they were sensitive to cell death on inhibition of p75NTR expression with transient transfection of small interfering RNA (siRNA). These findings suggest that p75NTR is necessary for survival and maintenance of ESCC tumors, providing us with a potential target for novel therapies.

Purpose: p75NTR is expressed in a stem/progenitor cell fraction of human normal esophageal epithelial cells. In this study, we investigated the expression and biological role of p75NTR in ESCC.

Experimental Design: The expression of p75NTR in 187 resected ESCC specimens was immunohistochemically investigated. The expression of p75NTR in 30 ESCC cell lines (KYSEs) was assessed by reverse transcription-PCR, immunocytochemistry, and flow cytometry. The p75NTR-bright and p75NTR-dim/negative cells were isolated from KYSE150 by magnetic beads and colony formation was investigated. The role of p75NTR in KYSEs was assessed by transient transfection of siRNA.

Results: p75NTR was expressed in 92 of 187 (49.2%) tumors. In well-differentiated tumors, positive staining was apparent in the first one to two layers from infiltrative margin of the tumors where most of the cells were actively proliferating. In moderately differentiated tumors, p75NTR was expressed in wider range from the margin of the tumors whereas p75NTR was diffusely distributed in poorly differentiated tumors. p75NTR was expressed in all examined KYSEs and the mean proportion of the p75NTR-bright fraction was 30.1%. The size of p75NTR-positive colonies was larger than that of p75NTR-negative colonies derived from KYSE150 (P < 0.0001). The purified p75NTR-bright cells formed p75NTR-positive large colonies more frequently than the p75NTR-dim/negative cells (P < 0.0001). Down-regulation of p75NTR expression by siRNA resulted in marked growth inhibition with induction of apoptosis.

Conclusions: Our findings suggest that p75NTR is necessary for survival and maintenance of ESCC tumors, providing us with a potential target for novel therapies.

Esophageal squamous cell carcinoma (ESCC) is a highly lethal malignancy with a 5-year survival rate of 20% to 30% after curative surgery (1–3). Thus, improved treatments derived from a better understanding of the biological basis of esophageal cancer cell growth are now emerging.

Recent observations using several malignancies have shown the identification of a limited number of cells with a remarkable self-renewal potential and extensive proliferation capacity (4–7). Most of these cells have been isolated from whole tumor cells based on the expression of markers that characterize the stem cells of the original normal tissues (8). These cells are exclusively responsible for the growth and propagation potential of the whole tumor (5, 6, 9) and they are thought to be a novel target for therapies (8, 10).

For human normal esophageal epithelial cells, a candidate stem/progenitor cell fraction is characterized by the expression of the low-affinity p75 neurotrophin receptor (p75NTR) in vitro...
(11, 12). These cells retain a slowly cycling and relatively immature phenotype and are capable of repopulating all known epithelial cell subsets (11).

p75NTR is a 75-kDa cell-surface receptor glycoprotein, which is a member of the tumor necrosis factor receptor superfamily (13) and involved in diverse cellular response, including cell proliferation, cell survival, and apoptosis, in neural and non-neural tissues (14), and various levels of its expression and function in several cancers have also been reported (15–21). However, with regard to the ESCC, neither the expression nor the function of p75NTR has yet been reported.

In this study, we first investigated the expression of p75NTR in 187 resected ESCC specimens and 30 ESCC cell lines (KYSes) by immunohistochemical staining and reverse transcription-PCR (RT-PCR). We then fractionated the p75NTR-bright and p75NTR-dim/negative cells from an ESCC cell line by magnetic cell sorting and investigated their colony-forming capacity. Finally, by inhibiting p75NTR expression using transient transfection of small interfering RNA (siRNA), we elucidated its biological role and possible use for therapeutic application in ESCC.

Materials and Methods

Patients and surgical specimens. A total of 187 patients with ESCC who underwent surgery at Kyoto University Hospital between 1990 and 2000 were examined. Of these 187 patients, 31 were female and 156 were male. The median age of the patients was 64 years with a range of 43 to 84 years. The median follow-up was 68.5 months with a range of 3 to 121 months. Information on gender, age, stage of disease, and histopathologic factors was abstracted from the medical records. All of the tumors were confirmed as ESCC by the clinicopathologic department of the hospital. All of the cases were classified according to the fifth edition of the pathologic tumor-node-metastasis (TNM) classification of 1997 (22). Of all 187 patients, 44 (23.5%) had T1, 49 (26.2%) had T2, 60 (32.1%) had T3, and 34 (18.2%) had T4. Written informed consent was obtained from the patients for surgery and to use resected samples for research (Institutional Review Board of Kyoto University, approval nos. 232 and G48).

Immunohistochemical staining. Tissues were fixed in 10% formalin, embedded in paraffin, and processed by standard methods. From each selected paraffin block, 4-μm serial sections were cut. These sections were deparaffinized with xylene and rehydrated through a graded alcohol series. Then the sections were autoclaved at 121°C in Target Retrieval Solution (Dako Cytomation, Kyoto, Japan) for 5 minutes and cooled to room temperature to unmask antigens. After washing in water, the sections were rinsed with PBS. Then immunostaining was done with Envision Plus kits/horseradish peroxidase/3,3′-diaminobenzidine (Dako Cytomation) as recommended by the supplier. Primary antibodies were diluted to 1:50 for anti-human p75NTR monoclonal antibody against p75NTR-extracellular domain (clone NGFR5; Dako Cytomation) or 1:100 for anti-human Ki67 monoclonal antibody (clone MIB1; Dako Cytomation) with 1% bovine serum albumin in PBS, and sections were incubated in a humidified atmosphere of 5% CO2 in the air.

Flow cytometry. Adherent cells were trypsinized, washed once in cold PBS, and −2 × 10^5 viable cells were resuspended in 50 μL of PBS with 0.02% sodium azide and 0.5% bovine serum albumin (staining buffer). For single staining before fluorescence-activated cell sorting analysis (Becton Dickinson, Inc., San Jose, CA), cells were incubated with 1 mg/mL of mouse anti-p75NTR (clone NGFR5). After washing with staining buffer, cells were resuspended in 50 μL of staining buffer and incubated with 1 mg/mL of FITC-conjugated goat anti-mouse immunoglobulin G (IgG) antibody. Both primary and secondary staining reactions were carried out for 15 minutes at room temperature. Nonspecific isotypematched antibodies were used as controls. Results were analyzed with Cell Quest software (Becton Dickinson).

Immunocytochemical staining. Cultured cells were fixed with 3.7% formaldehyde in PBS at room temperature for 30 minutes. Then immunocytochemistry was done with Envision Plus kits/horseradish peroxidase/3,3′-diaminobenzidine (Dako Cytomation) as recommended by the supplier. Primary antibodies were diluted to 1:50 for anti-human p75NTR monoclonal antibody (clone NGFR5; Dako Cytomation) or 1:100 for anti-human Ki67 monoclonal antibody (clone MIB1; Dako Cytomation) with 1% bovine serum albumin in PBS, and sections were incubated in a moist chamber overnight at 4°C. Counterstaining was carried out with Mayer’s hematoxylin, dehydrated, and mounted.

Cell fractionation. The p75NTR-positive and p75NTR-negative cells were separated with anti-mouse IgG antibody conjugated magnetic beads (Dynabeads M450, Dynal A.S. Co., Oslo, Norway) that had been incubated with saturating concentrations of mouse anti-p75NTR antibody (clone NGFR5; Dako Cytomation) at 4°C for 2 hours, then washed thrice with PBS(−). Monolayers of cultured cells were trypsinized, washed, and resuspended at a concentration of 1.0 × 10^7/mL in PBS with 0.1% bovine serum albumin. Then the cells were mixed with antibody-conjugated Dynabeads at a ratio of four particles per cell. The mixture was incubated at 4°C for 60 minutes with gentle agitation. Cells bound to Dynabeads (p75NTR-positive cells) were separated under a magnetic field using Dynal MPC-M (Dynal).

Western blot analysis. Cells were lysed in a sample buffer (2% SDS, 10% glycerol, 50 mmol/L Tris-HCl, pH 6.8) at room temperature. Cell lysate was sonicated and protein concentration was estimated by Bradford method with Bio-Rad Protein Assay Reagent (Bio-Rad Laboratories, Inc., Hercules, CA). After boiling for 2 minutes, cell
lysates (20 μg) were electrophoresed on 2% to 15% gradient polyacrylamide gel (Daichi Pure Chemicals, Tokyo, Japan) and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were blocked with 2% skim milk (Difco, Detroit, MI) in 0.1% Tween 20 in TBS (20 mmol/L Tris, 150 mmol/L NaCl, pH 7.6) for 1 hour at room temperature. A rabbit anti-p75NTR polyclonal antibody (G3323, Promega, Madison, WI) was used as the primary antibody against p75NTR intracellular domain. The membrane was subsequently incubated at room temperature for 1 hour with horseradish peroxidase–linked goat anti-mouse IgG antibody (EY Laboratories, Inc., San Mateo, CA; diluted 1:1,000). The final detection of specific proteins was carried out with enhanced chemiluminescence reagents (Amersham Biosciences Corp., Piscatway, NJ) and visualized on X-ray film.

**SiRNA transfection.** The sequences of siRNA targeting p75NTR used in this study were sense, 5'-CAGCUUGCAAGCAGAAAGTT-3', and antisense, 5'-CUUGCUGCCGACGACUUGT-3'. The nonspecific control siRNA was purchased from Dhamacon, Inc. (Lafayette, CO); the sequences were sense, 5'-AUUGUAUGCGAUCGCAGACUU-3', and antisense, 5'-GICUCCGCAUCGCUAAUUU-3'.

Cells were trypsinized and replaced into six-well dishes. After 24 hours, at a density of 20% of 50% confluency, the cells were transfected with siRNA using Oligofectamine reagent (Invitrogen) according to the instructions of the manufacturer. The final concentration of siRNA was 80 nmol/L per dish (nmol/L). Cells were used for analysis 72 hours after transfection.

**Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assays.** DNA staining with the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay was done with the DeadEnd Colorimetric TUNEL System (Promega) following the instructions of the manufacturer. At least 300 cells were counted in each section and the number of TUNEL-positive apoptotic cells per 100 tumor cells was expressed as an apoptotic index in percent.

**Statistical analysis.** P75NTR expression and clinicopathologic factors were analyzed by χ² analysis. The overall survival was defined as that from the date of operation to the date of patient death due to cancer. Kaplan-Meier method was used to generate the survival curve and data were analyzed by the log-rank test. Multivariate analysis was done with the Cox's proportional hazard model.

The data obtained from the colonogenic assays were analyzed by Student's t test, which compares whether the mean of a single variable differs between two groups. P < 0.05 was considered significant.

**Results**

**Expression of p75NTR in ESCC specimens.** We first examined the expression of p75NTR in ESCC by immunohistochemistry. As has been shown in several studies, p75NTR was expressed in the basal cells of the normal esophageal epithelium and the p75NTR immunoreactivity in the basal layer of normal epithelium was used as an internal positive control in every specimen (Supplementary Fig. S1).

Tumor was classified as p75NTR positive when >10% of the tumor cells were stained because the percentages of p75NTR staining–positive cells in each tumor were broadly distributed from 10% to 100% (Supplementary Fig. S2). Of the 187 resected ESCC specimens, 92 (49.2%) were positive for p75NTR expression and 95 (50.8%) were negative.

In well-differentiated cases, positive staining was apparent in the first one to two layers from the infiltrative margin of the tumors where most of the cells were actively proliferating (Fig. 1A-C). Areas exhibiting stratified squamous pearl formation were negative for p75NTR. In moderately differentiated cases, p75NTR was expressed in wider range from the margin of the tumors (Fig. 1D-F). p75NTR was diffusely distributed in poorly differentiated tumors (Fig. 1G-I).

The correlation between p75NTR expression and various prognostic factors such as pTNM pathologic classification, histopathologic grading, and stage grouping were investigated (Table 1). P75NTR expression correlated with negative lymph node metastasis (P = 0.004) and lower TNM staging.
There was no significant correlation between p75NTR expression and other factors such as age, gender, extent of the tumors, distant metastasis, histology, and tumor location.

Kaplan-Meier survival curve revealed that p75NTR expression correlated with a favorable outcome (Fig. 2). The univariate analysis showed that p75NTR expression was a favorable prognostic factor ($P = 0.013$; odds ratio, 1.874; 95% confidence interval, 1.144-3.068). The multivariate analysis showed that pT ($P = 0.002$) and pN ($P = 0.006$) were independent prognostic factors, but p75NTR expression ($P = 0.455$) was not.

**Expression of p75NTR in ESCC cell lines.** Next, we examined the expression of p75NTR in 30 ESCC cell lines. p75NTR mRNA was expressed in all examined KYSEs detected by RT PCR (Fig. 3A). Immunocytochemistry using an antibody against the extracellular domain of p75NTR showed various intensities of staining at the membrane sites of the cells (Fig. 3B). Our flow cytometric analysis revealed that a small number of cells strongly expressed p75NTR when compared with the control IgG staining, whereas weak expression was seen in a certain number of the cells (Fig. 3C and D). The mean proportion of the cells with intense expression of p75NTR in each KYSE was 30.1%, ranging from 0.5% to 91.8% (Supplementary Fig. S3).

Because p75NTR is reported to be expressed in a small number of the cells, which is important to long-term proliferation of normal esophageal keratinocyte (11), and also because p75NTR was expressed in the actively proliferating zone of ESCC tumors (Fig. 1), we next examined the p75NTR expression in colonies derived from an ESCC cell line. We studied KYSE150, in which p75NTR was intensely expressed in 8.9% of the cells detected by flow cytometry (Fig. 3D). There were various sizes of colonies derived from KYSE150 at 2 weeks after plating (Fig. 4A-a). p75NTR-positive cells were immuno- cytochemically detected in 118 of 194 (60.8%) colonies (Fig. 4A-b). The distribution of colony size in p75NTR-positive and p75NTR-negative colonies showed that p75NTR expression correlated with a larger colony size (Fig. 4C-a), with a mean diameter of 969 ± 513 and 414 ± 182 µm in p75NTR-positive and p75NTR-negative colonies, respectively ($P < 0.0001$). A high-power magnification of the photo of p75NTR-positive colony showed that although the p75NTR-positive cells were diffusely distributed in the p75NTR-positive colonies, the intensity was higher in the center of the colonies (Supplementary Fig. S4A). Immunocytochemical detection of Ki67 in 20 colonies derived from KYSE150 showed equally scattered distribution of Ki67-positive cells in the colonies without accumulation of the positive cells in the center of the colonies (Fig. S4B).

**Table 1. Relationship between p75NTR expression and clinicopathologic characteristics**

<table>
<thead>
<tr>
<th>Tissue type</th>
<th>Total cases (%)</th>
<th>p75NTR positive, n (%)</th>
<th>p75NTR negative, n (%)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;65 y</td>
<td>93 (49.7)</td>
<td>47 (51.1)</td>
<td>46 (48.4)</td>
<td>0.716</td>
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<tr>
<td>≥65 y</td>
<td>94 (50.3)</td>
<td>45 (48.9)</td>
<td>49 (51.6)</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>156 (83.4)</td>
<td>75 (81.5)</td>
<td>81 (85.3)</td>
<td>0.492</td>
</tr>
<tr>
<td>Female</td>
<td>31 (16.6)</td>
<td>17 (18.5)</td>
<td>14 (14.7)</td>
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<td>TNM</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>$T_1/T_2$</td>
<td>93 (49.7)</td>
<td>50 (54.3)</td>
<td>43 (45.3)</td>
<td>0.214</td>
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<tr>
<td>$T_3/T_4$</td>
<td>94 (50.3)</td>
<td>42 (45.7)</td>
<td>52 (54.7)</td>
<td></td>
</tr>
<tr>
<td>$N_0$</td>
<td>70 (37.4)</td>
<td>44 (47.8)</td>
<td>26 (27.4)</td>
<td>0.004</td>
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<tr>
<td>$N_1$</td>
<td>117 (62.6)</td>
<td>48 (52.2)</td>
<td>69 (72.6)</td>
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<tr>
<td>$M_0$</td>
<td>160 (85.6)</td>
<td>81 (88.0)</td>
<td>79 (83.2)</td>
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<tr>
<td>$M_1$</td>
<td>27 (14.4)</td>
<td>13 (12.0)</td>
<td>16 (16.8)</td>
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<tr>
<td>TNM stage</td>
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<td></td>
<td></td>
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<tr>
<td>I/II</td>
<td>96 (51.4)</td>
<td>55 (59.8)</td>
<td>41 (43.2)</td>
<td>0.023</td>
</tr>
<tr>
<td>III/IV</td>
<td>91 (48.6)</td>
<td>37 (40.2)</td>
<td>54 (56.8)</td>
<td></td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well differentiated</td>
<td>41 (21.9)</td>
<td>23 (25.0)</td>
<td>18 (19.0)</td>
<td>0.475</td>
</tr>
<tr>
<td>Moderately differentiated</td>
<td>103 (55.1)</td>
<td>51 (55.4)</td>
<td>52 (54.7)</td>
<td></td>
</tr>
<tr>
<td>Poorly differentiated</td>
<td>43 (23.0)</td>
<td>18 (19.6)</td>
<td>25 (26.3)</td>
<td></td>
</tr>
<tr>
<td>Location</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Upper</td>
<td>34 (18.2)</td>
<td>16 (17.4)</td>
<td>18 (19.0)</td>
<td>0.783</td>
</tr>
<tr>
<td>Middle/Lower</td>
<td>153 (81.8)</td>
<td>76 (82.6)</td>
<td>77 (81.0)</td>
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</table>

![Fig. 2. The association between p75NTR expression and patient prognosis. The cumulative survival rate of patients with immunohistochemically p75NTR-positive tumors was higher than that of patients with p75NTR-negative tumors.](www.aacnjournals.org)
fractionated KYSE150 based on p75NTR expression by magnetic cell sorting and assessed the colony formation of each fraction.

An average proportion of 5.5% and 80.4% of the cells were viably recovered as positive and negative fractions, respectively (Table 2), whereas an average proportion of 0.9% was nonspecifically separated by using magnetic beads not conjugated with anti-p75NTR antibody (data not shown). Western blot analysis and RT-PCR for p75NTR showed strong expression at both protein and mRNA levels in the positive fraction; however, weak expression was detected even in the negative fraction (Fig. 4B). A low intensity level of p75NTR expression was detected in the negative fraction by immunocytochemistry (Supplementary Fig. S5), indicating that the magnetic bead separation could not capture certain number of weakly positive cells. Based on these results, we named the positive and negative fractions as p75NTR-bright cells and p75NTR-dim/negative cells, respectively.

When the fractionated cells were plated and cultured, p75NTR was expressed in 178 of 217 (82.0%) colonies derived from p75NTR-bright fraction whereas 65 of 216 (30.1%) colonies derived from p75NTR-dim/negative fraction were positive for p75NTR. The summarized distribution of colony size in p75NTR-positive and p75NTR-negative colonies further showed the relationship between colony size and p75NTR expression (Fig. 4C-b and c). Both p75NTR-positive and p75NTR-negative cells were immunocytochemically detected in colonies derived from isolated p75NTR-bright fraction (Supplementary Fig. S6).

**Inhibition of p75NTR expression by siRNA induced apoptosis in an ESCC cell line.** Because the expression of p75NTR correlated with the proliferative fraction of ESCC tumors and also ESCC cell lines, we next examined whether p75NTR is necessary for maintaining proliferation potential of ESCC cell line.

At 72 hours after transient transfection of siRNA targeting p75NTR into KYSE150, the expression of p75NTR at protein level was successfully inhibited as shown by Western blot analysis (Fig. 5A), flow cytometry (Fig. 5B), and immunocytochemistry (Fig. 5C). The down-regulation of p75NTR expression was in parallel with the inhibition of cell growth as measured by the cell survival rate at 72 hours after siRNA transfection (Fig. 5D). The TUNEL assay showed a marked induction of apoptosis (Fig. 5E) with 15.5% apoptotic index in the siRNA-treated cells and 1.5% and 3.0% apoptotic indexes in the nontreated and nonspecific control siRNA-treated cells, respectively (Fig. 5F).

**Discussion**

In this study, we investigated the expression of p75NTR in a large number of ESCC specimens and found that 92 of 187 (49.2%) were positive for p75NTR. Its expression correlated with negative lymph node metastasis and associated with favorable prognosis, compatible with previous reports that the loss of p75NTR expression resulted in poor prognosis in several cancers (17, 19, 20, 24).

In p75NTR-positive ESCC tumors, p75NTR was expressed in the first one to two layers from the infiltrative margin of well-differentiated tumors, where most of the cells were actively proliferating, whereas p75NTR was diffusely distributed in the poorly differentiated tumors, suggesting that p75NTR is expressed in the actively proliferating, undifferentiated cell component of each tumor. Recently, the intratumor heterogeneity with respect to proliferation and differentiation has been shown and a small number of cells that possess stem-cell properties have been identified in several solid tumors (5–7). These cells could exclusively generate tumors in mice or form colonies in plate after purification (5, 6, 9). Taken together with our in vitro study that p75NTR was intensely expressed in a small number of cells in ESCC cell lines and that p75NTR-bright cells have a high colony-forming efficacy, it is possible...
that p75NTR is intensely expressed in the tumorigenic fraction of ESCC.

The p75NTR-positive colonies established from the isolated p75NTR-bright cells contained both p75NTR-positive and p75NTR-negative cells, similar with the expression in colonies derived from nonfractionated KYSE150, suggesting that the p75NTR-bright cells gave rise to p75NTR-negative cells. In addition, the immunochemical detection for Ki67 showed that not all the p75NTR-bright cells were proliferating in the colonies and not all the p75NTR-positive cells in the p75NTR-diffusely positive area in ESCC tumors were proliferating, indicating that some of p75NTR-positive cells were in the quiescent state of cell cycle although they showed high colony-forming capacity after isolation. Those are similar with the characteristics of p75NTR-positive cells in normal esophageal epithelial cells. Most of the p75NTR-positive cells in cultured normal esophageal epithelial cells are slowly cycling; however, they begin active proliferation rapidly after isolation and then give rise to p75NTR-negative cells to repopulate the original proportion of them. It is possible that the number of p75NTR-bright cells was regulated by the frequency of generating p75NTR-dim/negative cells and by the frequency of self-amplification in p75NTR-bright cell fraction in ESCC. Further evaluation of their characteristics, such as asymmetrical cell division, self-renewal capacity, and in vivo tumorigenic capacity, may provide us with a more detailed understanding of the relationship between p75NTR expression and stem-cell properties in ESCC.

Our study showed that inhibition of p75NTR expression using transient transfection of siRNA resulted in a marked inhibition

### Table 2. Cell fractionation based on p75NTR expression

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Pre-fractionation (C2)</th>
<th>Positive fraction (C2)</th>
<th>Negative fraction (C2)</th>
<th>Total recovery (%)</th>
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<tbody>
<tr>
<td>1</td>
<td>1.8 x 10^7</td>
<td>7.6 x 10^5 (4.2%)</td>
<td>1.63 x 10^7 (90.6%)</td>
<td>94.8</td>
</tr>
<tr>
<td>2</td>
<td>3.9 x 10^7</td>
<td>2.6 x 10^6 (7.7%)</td>
<td>2.6 x 10^7 (66.7%)</td>
<td>73.4</td>
</tr>
<tr>
<td>3</td>
<td>2.09 x 10^7</td>
<td>9.9 x 10^5 (4.7%)</td>
<td>1.69 x 10^7 (80.7%)</td>
<td>85.4</td>
</tr>
<tr>
<td>4</td>
<td>1.3 x 10^7</td>
<td>6.6 x 10^5 (5.1%)</td>
<td>1.07 x 10^7 (82.3%)</td>
<td>87.4</td>
</tr>
<tr>
<td>5</td>
<td>2.2 x 10^7</td>
<td>1.5 x 10^6 (6.8%)</td>
<td>1.8 x 10^7 (81.8%)</td>
<td>88.6</td>
</tr>
<tr>
<td>Mean ± SD (%)</td>
<td></td>
<td>5.5 ± 1.2</td>
<td>80.4 ± 8.6</td>
<td>85.9 ± 7.8</td>
</tr>
</tbody>
</table>

Fig. 4. p75NTR expression in colonies derived from KYSE150. A, a, crystal violet staining of colonies derived from KYSE150 at 2 weeks after plating. There were various sizes of colonies. b, p75NTR expression in colonies derived from KYSE150. There were p75NTR-positive and p75NTR-negative colonies. p, p75NTR-positive colony; n, p75NTR-negative colony. B, a, RT-PCR for p75NTR mRNA in p75NTR-positive and p75NTR-negative cell fractions separated from KYSE150 using magnetic beads. PF, positive fraction; NF, negative fraction. G3PDH mRNA was used as an internal standard. b, Western blot analysis for p75NTR protein in p75NTR-positive and p75NTR-negative cell fractions separated from KYSE150 using magnetic beads. The amount of β-actin in the blot confirms equivalent protein loading for each lane. C, a, distribution of colony size in p75NTR-positive and p75NTR-negative colonies derived from KYSE150. The diameter and p75NTR positivity were examined in 194 colonies. b, distribution of colony size in p75NTR-positive and p75NTR-negative colonies derived from p75NTR-positive cell fraction separated from KYSE150. The diameter and p75NTR positivity were examined in 217 colonies. c, distribution of colony size in p75NTR-positive and p75NTR-negative colonies derived from p75NTR-negative cell fraction separated from KYSE150. The diameter and p75NTR positivity were examined in 218 colonies.
of cell growth with induction of apoptosis. TUNEL staining revealed that apoptosis was induced in >15.5% of KYSE150 after treatment with siRNA, despite only 8.9% of the cells being identified as p75NTR-bright fraction. Based on the results that p75NTR expression was completely inhibited by transient transfection of siRNA, it is suggested that even weakly expressed p75NTR has antiapoptotic role whereas the brightness of p75NTR expression correlated to the colony-forming capacity.

The induction of apoptosis by inhibition of p75NTR expression is compatible with previous reports that the survival and proliferation are stimulated through p75NTR in breast cancer cells (25–27). On the other hand, it is incompatible with the reports that p75NTR suppresses growth of prostate and bladder cancer cell lines (16, 17). The similar diverse response, such as cell survival and apoptosis, is mediated by p75NTR in different types of cells in neuronal tissues (14) and one proposed implication is that p75NTR mediates various biological effects depending on whether tyrosine kinase A is coexpressed or not. p75NTR acts both as a Trk coreceptor and as an autonomous signaling molecule (28), and it has been reported that costimulation of tyrosine kinase A and p75NTR results in cell survival, whereas when p75NTR is expressed in isolation, ligand binding triggers cellular apoptosis (29–33).

Based on our unpublished results that NGF and tyrosine kinase A are expressed in most of ESCC cell lines, it is possible that cell survival is mediated by p75NTR and tyrosine kinase A in ESCC cells, similar with the reported model in breast cancer cells (25). More detailed investigation of the correlation between p75NTR and other molecules such as Trk receptors in ESCC may provide us with the basis to develop the novel therapies targeting p75NTR in ESCC patients.

In conclusion, we investigated the clinicopathologic significance of p75NTR in 187 resected ESCC specimens and found that ~50% of ESCC expressed p75NTR. Our investigation using ESCC cell lines showed that p75NTR was expressed in the cells with high colony-forming capacity but they were sensitive to cell death on inhibition of p75NTR expression with transient transfection of siRNA. These findings suggest that p75NTR is expressed in the cells with high colony-forming capacity and is necessary for survival and maintenance of ESCC, providing us with a potential target for novel therapies.

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Tomoyuki Okumura, Shigeru Tsunoda, Yukiko Mori, et al.


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