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Biochemical and Biophysical Research Communications 358 (2007) 226–232
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# Induction of apoptosis by an inhibitor of EGFR in neuroblastoma cells

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Received 11 April 2007
Available online 27 April 2007

**Abstract**

We aimed to examine the expression of EGFR in neuroblastoma tissues and to investigate the antitumor activity of a selective EGFR-tyrosine kinase inhibitor, gefitinib, on neuroblastoma. The expression of EGFR was detected in each of two tumor tissues by immunohistochemistry and eight of 10 cell lines by Western blotting. Gefitinib inhibited EGFR-phosphorylation and *in vitro* cell growth ($IC_{50}$: approximately 1.2 μM), and a high concentration of gefitinib (20–30 μM) induced apoptosis *in vitro*. This is the first report that EGFR protein is expressed on the cell surface in neuroblastoma tissues and in cell lines. We also demonstrated an EGFR inhibitor induced apoptosis on neuroblastoma cells. Our results suggest the feasibility of targeting EGFR as a novel strategy against neuroblastoma.


*Keywords:* Neuroblastoma; Epidermal growth factor receptor; Gefitinib; Apoptosis

Neuroblastoma (NB) is the most common extracranial solid tumor in children and is characterized by a wide range of clinical behaviors, from spontaneous regression to rapid progression with a fatal outcome [1]. The outcome for children older than 1 year with stage four NB and *MYCN* amplification remains poor even after high-dose chemotherapy followed by stem-cell transplantation. Therefore, new therapeutic approaches are needed against aggressive NB.

Recently, some molecular-targeted anticancer drugs, which specifically inhibit protein tyrosine kinase receptors, have been developed and used clinically [2], i.e., imatinib for chronic myeloblastic leukemia, and gefitinib for non-small cell lung cancer (NSCLC). Imatinib has been shown to have antitumor activity against NB *in vitro* and *in vivo* [3]. Imatinib inhibits the signal transduction pathway of platelet-derived growth factor receptor and/or SCF/c-Kit, and suppresses tumor progression in NB.

Gefitinib selectively inhibits epidermal growth factor receptor (EGFR)-tyrosine kinase [4]. EGFR has a role in cellular proliferation, inhibition of apoptosis and angiogenesis, and is abnormally activated in many epithelial tumors including NSCLC, head and neck cancer, and breast cancer [5]. Also, in NB, EGFR signaling has been associated with cell cycle progression and cellular proliferation. Mirkin et al. [6] showed that EGF increased the cell proliferation rate of NB cells, and that some gangliosides inhibited EGFR phosphorylation and suppressed cell proliferation. Ho et al. [7] have also demonstrated that EGF increases cell number of NB, and stimulated cell cycle progression in NB. They also demonstrated that gefitinib inhibited the phosphorylation of EGFR and the proliferation of NB cells.

The goal of this study was to investigate the expression of EGFR protein on the cell surfaces in NB tissues and the apoptotic effect of gefitinib against NB cell lines. To determine the expression of EGFR protein, we used two tumor tissues and 10 cell lines. We also examined the effect of a combination of gefitinib and cisplatin on the growth of NB cells.

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doi:10.1016/j.bbrc.2007.04.124

## Materials and methods

*Tumor tissues, cell lines, and cell culture.* NB tissues were obtained from patients SI and TK in 1982 and 1992, respectively. NB cell lines were established previously from the same patients (designated KP-N-SIFA and KP-N-TK, respectively [8]). NB was confirmed by clinical and histological examinations. EGFR expression was examined in tumor tissues that had been fixed in 10% formalin and embedded in paraffin. The other eight human NB cell lines used (KP-N-SILA, KP-N-SKE, MP-N-TS, KP-N-YS, KP-N-NY, KP-N-AYR, KP-N-RTBM1, and KP-N-HN) were also established in our laboratory from either surgically resected tumors or bone marrow aspirations. Human epidermoid carcinoma (A431) cells were used as a positive control for EGFR expression. All cell lines were maintained as described previously [9].

*Reagents*. Gefitinib was a kind gift of AstraZeneca (Macclesfield, UK). Stock solutions were prepared in DMSO and stored at −80 °C. EGF was obtained from Invitrogen (Carlsbad, CA). Stock solutions were prepared in 1% FBS-RPMI and stored at −80 °C. Cisplatin was obtained from Calbiochem (Darmstadt, Germany).

*Real-time quantitative RT-PCR (TaqMan)*. Total RNA was extracted from cell lines using the QIAamp RNeasy Protect Mini kit (Qiagen, GmbH, Germany), and reverse transcribed to synthesize cDNA using the Superscript™ First-strand Synthesis System for RT-PCR (Invitrogen), according to the manufacturer's instructions. Quantitative real-time RT-PCR was carried out with the TaqMan, Master Mix (Applied Biosystems, Foster, CA) and analyzed using the ABI-PRISM 7300 Sequence Detection System. The primer and probe sequences for *EGFR* were obtained from the previous report [10], and those for *GAPDH* were designed, respectively, using Primer Express ver. 1.5 (Applied Biosystems). All primers pairs and the probe were designed so that either one of the primers or the probe could span an exon–exon boundary. Specific primers and probes were as follows. For *EGFR*, 5′-CCACCTGTGCCATCCAAA CT-3′ (forward); 5′-GGCGATGGACGGGATCTT-3′ (reverse); probe 5′-FAM CCAGGTCTTGAAGGCTGTCCAACGAAT-TAMRA-3′. For *GAPDH*, 5′-TGAAGGTCGGAGTCAACGG-3′ (forward); 5′-AGAGTTAA AAGCAGCCCTGGTG-3′ (reverse); probe 5′-VIC-TTTGGTCGTATT GGGCGCCTGG-TAMRA-3′. All expression levels were normalized to *GAPDH*, and the ratios of the fetal brain (Clontech Labs, Tokyo, Japan) were used as calibrators of 1.0. mRNA expression levels were calculated as the average of two measurements.

*Immunofluorescence.* NB cells were cultured on coverslips, fixed in methanol, rehydrated in PBS, incubated with EGFR monoclonal antibody (DAKO, Glostrup, Denmark), incubated with fluorescein isothiocyanate-conjugated anti-mouse IgG (Cappel Products, Aurora, OH), and observed as previously described [9].

*Histochemistry and immunohistochemistry.* Five micrometer sections made from paraffin-embedded samples were stained with hematoxylin and eosin. Adjacent sections were used for immunohistochemistry. Sections were immunostained with a dextran polymer conjugate (EnVision+ system, DAKO, Glostrup, Denmark) as described previously [9].

*In vitro cell growth and cytotoxicity assay.* Cell viability was measured using 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium monosodium salt (WST-8) reagent (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's protocol. KP-N-TK and KP-N-SIFA cell lines were used to study the proliferative effect of EGF and the inhibitory effect of gefitinib. KP-N-TK cells were plated at a density of $7.5 \times 10^3$ per well in 10% FBS-RPMI in triplicate into 96-well cell plates overnight. After 24 h, cells were treated with different concentrations of gefitinib or DMSO in the absence or presence of EGF (100 ng/mL) in 2% FBS-RPMI, and cultured for an additional 96 h. KP-N-SIFA cells were plated at a density of $2.0 \times 10^4$ per well in 10% FBS-RPMI in triplicate into 96-well cell plates. After 24 h, cells were treated with gefitinib or DMSO for an additional 96 h in 10% FBS-RPMI, since KP-N-SIFA cell line cannot survive in serum starvation condition. Viability was assayed every 24 h after treatment of gefitinib. To assess the effect of a combination of gefitinib and cisplatin on growth, the drugs were administered according to Magné's report [11]. In brief, KP-N-TK and KP-N-SIFA cells were plated in triplicate into 96-well cell plates, incubated for 24 h, treated with DMSO or 1 μM of gefitinib for a further 48 h in 10% FBS-RPMI, exposed to different concentrations of cisplatin with DMSO or 1 μM of gefitinib for a further 48 h and examined for viability.

*Immunoblot analyses.* After the indicated periods, cells were solubilized in RIPA buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 1% Nonidet P-40, 0.5% deoxycholate, and 0.1% sodium dodecyl sulfate). Floating cells were also collected. Total protein (50–100 μg) was separated on an SDS–PAGE. Western blot analysis was performed using various antibodies (anti-EGFR monoclonal antibody (BD Bioscience, San Jose, CA), β-actin antibody (Sigma–Aldrich Japan, Tokyo, Japan), anti-caspase-3 antibody (BD Biosciences), anti-cleaved caspase-3 antibody (Cell Signaling Technology, Danvers, MA), and anti-caspase-7 antibody (Cell Signaling Technology) as described previously [8].

*Immunoprecipitation.* Lysates were prepared as described above, incubated with the appropriate amount of anti-EGFR antibody and protein A/G-plus agarose (Santa Cruz Biotechnology, Santa Cruz, CA), and separated by SDS–PAGE. Western blot analysis was performed using anti-phosphotyrosine antibody (PY20, BD Bioscience) or anti-EGFR antibody as described previously [9].

*Terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) assay.* Apoptosis was determined using a MEBSTAIN Apoptosis Detection Kit Direct (MBL, Nagoya, Japan) according to the manufacturer's protocol. Cells were plated onto 100-mm dishes, treated with gefitinib or DMSO in 10% FBS-RPMI for 96 h. The number of TUNEL-positive cells was calculated using Cell Quest software (Nippon Becton–Dickinson, Tokyo, Japan) as described previously [9].

*Statistical analysis.* Values are expressed as the means ± standard deviation (SD). The results of the apoptosis assay were compared with an ANOVA and a post hoc Fisher's PLSD-test to correct for multiple comparisons. The Effects on cell proliferation of gefitinib in combination with cisplatin were compared with Student's *t*-test. A difference between populations was judged significant at confidence levels greater than 95% ($P < 0.05$).

## Results

### *Expression of EGFR in NB clinical tissues and cell lines*

EGFR immunoreactivity was diffuse in both NB tissues (TK and SI) (Fig. 1A and C). Two of the 10 NB cell lines (KP-N-TK and KP-N-SIFA, originating from patients TK and SI, respectively) were selected to see if EGFR expression could be detected on the cell surfaces by immunofluorescence. Over 90% of KP-N-TK cells and 50% of KP-N-SIFA were stained (Fig. 1E and F). Western blotting showed that 8 of 10 cell lines, including KP-N-TK and KP-N-SIFA, expressed EGFR protein at various levels, although the levels were lower than the level in the A431 cells (Fig. 1H). In the NB cell lines the mRNA expression level of *EGFR* relative to *GAPDH*, *EGFR*/*GAPDH* ratio ranged from 0.005 to 1.57 with a median of 0.24, compared with that of fetal brain as calibrators of 1.0 (Fig. 1I). The expression level of *EGFR* in A431 was around 40 times higher than that in fetal brain. The *EGFR*/*GAPDH* ratio in KP-N-TK and KP-N-SIFA were 1.57 and 0.24, respectively. Comparison of the data in Fig. 1H and I showed the EGFR protein expression levels were well correlated to those of *EGFR* mRNA. The expression of EGFR had no apparent correlation with *MYCN* amplification status.

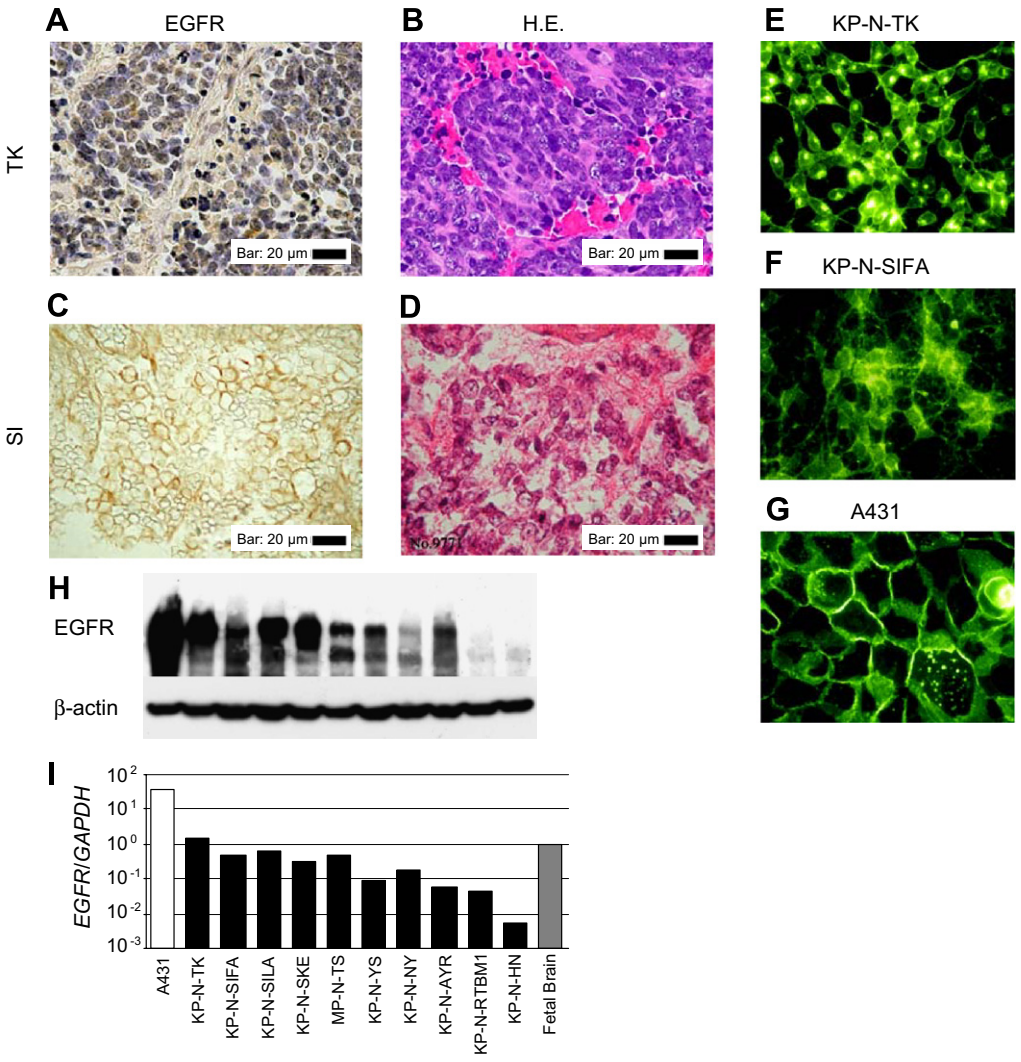


Fig. 1. Expression of EGFR on two NB tissues and cell lines. (A–D) Hematoxylin and eosin staining and immunohistochemical staining of EGFR in NB tissues. (E–G) Immunofluorescence staining of two NB cell lines (KP-N-TK and KP-N-SIFA) with anti-EGFR antibody. The A431 cell line was used as a positive control. (H) Western blot analysis of EGFR in 10 NB cell lines. Lysates were prepared and immunoblotted for anti-EGFR antibody. β-Actin was used to check for equal protein loading. (I) Quantitative real-time RT-PCR analysis of *EGFR* mRNA expression in 10 NB cell lines. All expression levels were normalized to *GAPDH*, and the ratios of the fetal brain were used as calibrators of 1.0.

*Inhibition of EGFR phosphorylation by gefitinib in NB cell lines*

Next, the ability of gefitinib to inhibit EGFR phosphorylation was examined with the KP-N-TK cell line. The baseline phosphorylation of EGFR was absent in this cell line cultured in serum-free medium. However, treatment with EGF (100 ng/ml) for 5 min induced tyrosine phosphorylation of EGFR. This effect was decreased in a dose-dependent manner by pretreatment with gefitinib (0.01–30 μM) before the addition of EGF. Inhibition was clearly evident at gefitinib concentrations as low as 0.01 μM (Fig. 2A).

*Inhibition of NB cell growth by gefitinib*

KP-N-TK cells growing in 2% FBS-RPMI were selected to test the effect of EGF on growth. In the absence of gefitinib, 100 ng/ml EGF caused a 1.3-fold increase in cell number by day 4. When cells in the linear growth phase were grown in the presence of EGF and gefitinib at concentrations from 0.1 to 30 μM, cell proliferation was inhibited in a dose-dependent manner (Fig. 2B). The $IC_{50}$ value of gefitinib was 1.2 μM (Fig. 2C). When KP-N-TK and KP-N-SIFA cells were treated with gefitinib in 10% FBS-RPMI, the proliferation was also inhibited in a dose-dependent manner (Fig. 2D). Under these conditions, the $IC_{50}$ values of gefitinib were 10 and 12 μM (Fig. 2C and E). These $IC_{50}$ values were about 10 times higher than those obtained with cells cultured under serum starvation condition.

*Induction of apoptosis on NB cell by gefitinib*

Treatment of tumor cells with gefitinib for 96 h induced apoptosis in a dose-dependent manner. Induction of apop-

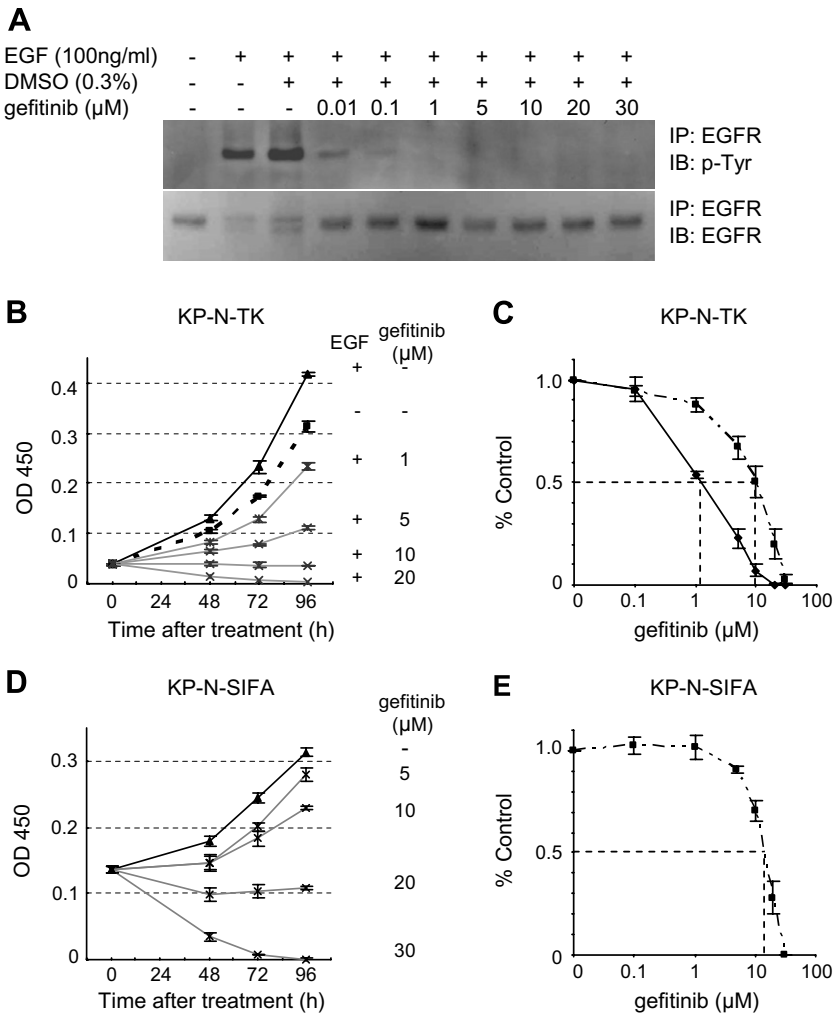


Fig. 2. Effect of gefitinib on NB cell lines. (A) Gefitinib inhibited EGFR phosphorylation at concentrations as low as 0.01 μM. Cells were placed in 2% FBS-RPMI for 24 h and then treated with the indicated concentrations of gefitinib for 60 min or DMSO. Cells were then stimulated (+) or not (−) with EGF for 5 min, lysed, and immunoprecipitated with anti-EGFR antibody. Immunoprecipitates were eluted, separated on a SDS–PAGE, and probed with anti-phosphotyrosine (p-Tyr) antibody (top). The blot was then stripped and reprobed with anti-EGFR antibody (bottom). IP, immunoprecipitation; IB, immunoblott. (B) Cell growth assay for KP-N-TK. Cell viability was determined by WST-8 assay. KP-N-TK cells were seeded and allowed to attach for 24 h, and cultured in 2% FBS-RPMI with 100 ng/ml EGF containing serial dilutions of gefitinib or DMSO for 96 h in triplicate cultures. The broken line shows the growth curve of cells cultured in 2% FBS-RPMI without EGF or gefitinib. Values are the mean of results from three wells from one of three similar experiments; bars, ±SD. (C) $IC_{50}$ value of the antiproliferation effect of gefitinib for 96 h. The $IC_{50}$ values of gefitinib for KP-N-TK cells cultured in 2% FBS-RPMI with 100 ng/ml EGF were 1.2 μM (solid line). The $IC_{50}$ value of gefitinib for KP-N-TK cells cultured in 10% FBS-RPMI was 10 μM (broken line). (D,E) Cell growth assay for KP-N-SIFA. KP-N-SIFA cells were seeded and allowed to attach for 24 h, and cultured in 10% FBS-RPMI containing serial dilutions of gefitinib or DMSO for 96 h in triplicate cultures. Values are the mean of results from three wells from one of three similar experiments; bars, ±SD. The $IC_{50}$ value of gefitinib for KP-N-SIFA cell in this condition was 12 μM.

tosis was significantly greater at 30 μM gefitinib than at 10 and 20 μM ($P < 0.001$, respectively) (Fig. 3A). Similarly, Western analysis revealed that 96 h treatment with gefitinib at 30 μM induced activation of caspases-3 and -7 in KP-N-TK and KP-N-SIFA cells (Fig. 3B). The activation of caspases-3 and -7 appeared after 12–24 h treatment of gefitinib and progressed until 72–96 h (Fig. 3C). These results suggest that growth inhibition of KP-N-TK and KP-N-SIFA cells by gefitinib at a concentration equal to $IC_{50}$ (10 μM) was due to cytostasis, whereas growth inhibition by gefitinib at a concentration greater than $IC_{50}$ (30 μM) was due to apoptosis.

### *Effects of a combination of gefitinib and cisplatin on cell proliferation*

Exposing the KP-N-TK and KP-N-SIFA cell lines to 1 μM gefitinib prior to and in combination with cisplatin slightly but significantly increased the cytotoxicity of cisplatin. A combination of 1 μM gefitinib and cisplatin at concentrations of 0.01, 0.1, 1, and 10 μM reduced the viability of KP-N-TK cells by 4%, 14%, 15%, and 13%, respectively, and reduced the viability of KP-N-SIFA cells by 19%, 18%, 10%, and 0%, respectively (Fig. 4).

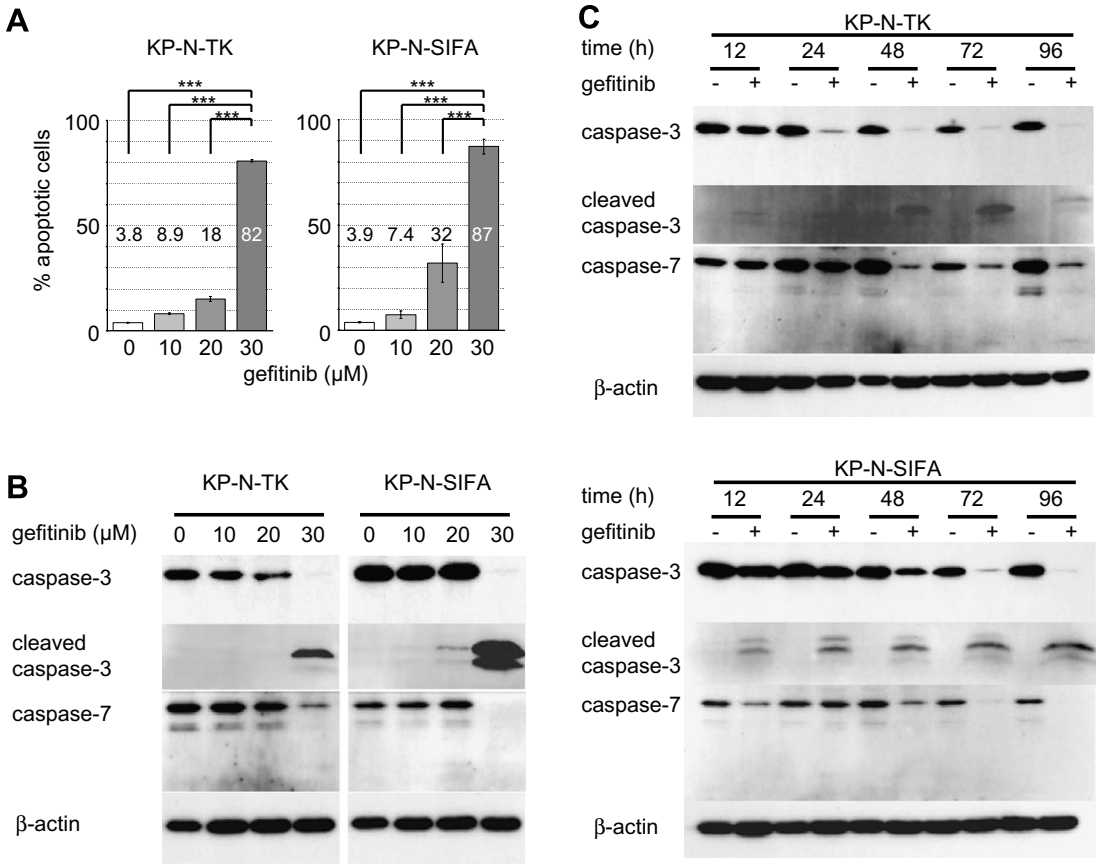


Fig. 3. Induction of apoptosis by gefitinib in two NB cell lines. (A) TUNEL assay. Cells were stained with fluorescein isothiocyanate-dUTP after 96 h of culture with gefitinib (closed gray columns), or 0.3% DMSO (open white columns), and examined by flow cytometry. The graphs show percentages of total apoptotic cells. Values are the mean of three independent experiences; bars, ±SD. *P* values relative to 30 μM gefitinib (***$P < 0.001$). (B) Cleavages of caspase-3 and caspase-7. NB cells were placed in 10% FBS-RPMI for 24 h and then treated with the indicated concentrations of gefitinib for 96 h. Control cells received drug vehicle: DMSO, final concentration 0.3%. Lysates were prepared and immunoblotted for various primary antibodies. β-Actin was used to check for equal protein loading. (C) Time-course of cleavages of caspase-3 and caspase-7 of KP-N-TK (upper) and KP-N-SIFA (lower) cells. NB cells were placed in 10% FBS-RPMI for 24 h and then treated with 30 μM gefitinib or DMSO for the times indicated. Lysates were prepared and immunoblotted as described above.

## Discussion

Previous reports that EGFR proteins are expressed in some NB cell lines, and that EGFR signaling mediates the proliferation of NB cells [6,7] prompted us to evaluate gefitinib, an EGFR-tyrosine kinase inhibitor, as a treatment for NB. Consistent with the previous reports, we found that EGFR is expressed on the surface of NB tumor cells and that EGF phosphorylate EGFR in a NB cell line, and increased the rate of proliferation of NB cells.

Gefitinib inhibited the growth of the two NB cell lines in a dose-dependent manner. The $IC_{50}$ values (10–12 μM) were close to those for head and neck cancer, lung cancer, breast cancer and malignant rhabdoid tumor cell lines under similar experimental conditions [9,12,13]. However, at much lower concentrations (0.01 μM), gefitinib was able to inhibit EGFR phosphorylation in one of the above NB cell lines (KP-N-TK). At concentrations greater than 1 μM, gefitinib is thought to have effects other than inhibition of EGFR [4]. When KP-N-TK cells were cultured under serum starvation conditions (2% FBS) to exclude endogenous growth factors, the $IC_{50}$ value decreased to 1.2 μM. This result suggests that gefitinib is able to suppress the growth of the NB cells by targeting EGFR.

Gefitinib also induced apoptosis on the two NB cell lines in a dose-dependent manner. Several pathways have been reported to be involved in the induction of apoptosis by gefitinib. Chang et al. showed that gefitinib treatment induced a marked increase of Fas protein expression, and activation of caspases-2, -3, and -8 in human lung adenocarcinoma A549 cells [14]. They suggested that the Fas/Fas-ligand death signaling pathway and the subsequent activation of the caspase-8/caspase-3 cascade, play a major role in gefitinib-induced apoptotic cell death. Gefitinib also induced Fas-mediated apoptosis in acinic cell adenocarcinoma [15]. Members of the tumor necrosis factor receptor super family, e.g., Fas, activate caspase-8. Activated caspase-8 leads to activation of pro-caspase-3 and initiation of the final pathway to apoptosis. In NB, caspase-8 expression is often lost in high-risk tumors [16]. In agreement with this finding, caspase-8 mRNA, as detected by RT-PCR was not detected in KP-N-TK, and was very low in

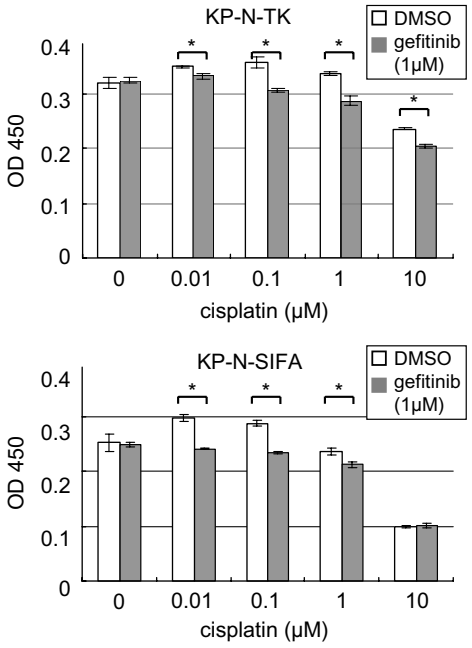


Fig. 4. Effects on cell proliferation of gefitinib in combination with cisplatin. Cells were seeded and allowed to attach for 24 h, and cultured in 10% FBS-RPMI with 1 μM gefitinib or control DMSO for 48 h. Then, cells were exposed to different concentrations of cisplatin with 1 μM of gefitinib or DMSO for a further 48 h. Viability was assayed 120 h after cell seeding by WST-8 assay. Values are the mean of results from three wells from one of three similar experiments; bars, ±SD, $^{*}P < 0.05$.

KP-N-SIFA (data not shown). Gefitinib might induce apoptosis in the NB cells in some way other than the death receptors pathway.

In previous reports [9,14,15] and our results, the concentrations of gefitinib needed to induce apoptotic cell death (20–30 μM) were higher than the concentrations needed to inhibit EGFR phosphorylation and cell proliferation. Therefore, it seems that gefitinib induces apoptosis not simply by blocking the tyrosine kinase activity of EGFR, but by being involved in multiple mechanisms. Our finding that higher concentrations of gefitinib were needed to induce cytostatic and cytotoxic effects in the NB cells than to inhibit the phosphorylation of EGFR maybe because EGFR expression levels in the NB cells are low. Currently, gefitinib is approved for oral administration at a dosage of 250 mg/day for NSCLC. In patients that received gefitinib orally at doses of 225 and 400 mg/day for 14 days, the plasma concentration of gefitinib ranged from 0.43 to 1.29 μM and from 0.86 to 2.94 μM, respectively [17]. Therefore, oral doses of 250 mg gefitinib per day are unlikely to induce apoptosis, although they are expected to have a cytostatic effect.

A combination of gefitinib and cisplatin was found to reduce cell viability by 5–15% more than cisplatin alone. In a variety of human cancer cell lines, gefitinib in combination with various cytotoxic drugs, including cisplatin was found to have an additive or synergistic antitumor effect [10,18,19]. Cisplatin was found to induce the phosphorylation of EGFR and Akt activation in a dose-dependent manner in human NSCLC cell [20]. The results of this study suggest that cisplatin activates EGFR signaling, and thus increases the sensitivities of gefitinib.

In conclusion, our results demonstrate that gefitinib has antitumor effects on NB cells and induced apoptosis *in vitro*. Our results suggest the feasibility of using EGFR targeting therapy as a novel strategy against NB.

## Acknowledgment

We thank Ryoko Murata for her secretarial assistance.

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