Antitumor Activity of Gefitinib in Malignant Rhabdoid Tumor Cells

In vitro and In vivo

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ABSTRACT

Purpose: Malignant rhabdoid tumor (MRT) is a rare and highly aggressive neoplasm of young children. Current treatments have had only limited success. Epidermal growth factor receptor (EGFR) was found recently to be expressed on MRT cell lines. Gefitinib (trade name Iressa) is an oral and selective EGFR-tyrosine kinase inhibitor and has been demonstrated to be effective in inhibiting the proliferation of cancer cells in vitro as well as in clinical trials. This encouraged us to examine the antitumor effects of gefitinib on MRT cells in vitro and in vivo.

Experimental Design: The expression of EGFR in two MRT tumors and two MRT cell lines (MP-MRT-AN and KP-MRT-NS), established from these two tumor tissues, was examined by immunohistochemistry, immunofluorescence, and immunoblot. The effect of gefitinib on EGFR phosphorylation was examined by immunoblot. The effects of gefitinib on cell growth and apoptosis were examined by cell growth assay and terminal deoxynucleotidyl transferase-mediated nick end labeling assay. The in vivo effect of gefitinib was assessed in athymic mice that had been xenografted with MRT cells.

Results: The expression of EGFR was detected in both tumor tissues and cell lines. Gefitinib inhibited EGFR-phosphorylation (IC50 < 0.1 μmol/L) and in vitro cell growth (IC50 = approximately 10–12 μmol/L), and a high concentration of gefitinib (20 μmol/L) induced apoptosis in vitro (MP-MRT-AN, 42.9% and KP-MRT-NS, 47.2%). Furthermore, gefitinib at 150 mg/kg had a cytostatic effect on established MRT xenografts (MP-MRT-AN, P = 0.039 and 0.0014; and KP-MRT-NS, P = 0.048 and 0.0086).

Conclusions: Our results demonstrate that gefitinib has antitumor effects in MRT cells in vitro and in vivo and, thus, has promise as a novel and therapeutic strategy for MRT.

INTRODUCTION

Malignant rhabdoid tumor (MRT) is a rare and extremely aggressive malignant tumor in childhood. It was initially described as an unfavorable histologic type of sarcomatous renal tumor, a variant of Wilms’ tumor (1). Subsequently, MRT was reported to also arise from extrarenal sites, including neck, heart, chest wall, liver, pelvis, and extremities (2, 3). Despite significant advances in treatment outcome of other pediatric tumors over the past 30 years, the overall survival of renal MRT was estimated at only 26% (4). Of particular note, only 9.7% of infants that were diagnosed before the age of 12 months were alive 4 years after diagnosis (4). Therefore, new therapeutic approaches are needed.

Epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase and is expressed in a wide variety of epithelial malignancies including non-small-cell lung cancer, head and neck cancer, and breast cancer (5). EGFR activation promotes tumor growth by increasing cell proliferation, motility, or angiogenesis, and by blocking apoptosis (6). Gefitinib (trade name Iressa) is an oral and selective EGFR-tyrosine kinase inhibitor that blocks the signal transduction pathways implicated in proliferation of cancer cells (7). Gefitinib has been demonstrated to be effective in athymic mice studies (8–10) as well as in Phase II clinical trials for non-small-cell lung cancer (11).

About 13 MRT cell lines have been documented (12, 13). The expression of EGFR on MRT cell lines was shown by immunohistochemistry and cell growth inhibition by anti-EGFR antibody (14). However, the expression of EGFR on MRT tissues and the phosphorylation of EGFR by epidermal growth factor (EGF) on MRT cell lines have never been examined. We established two MRT cell lines from two MRT patients (13, 15). In this study, we confirm the genetic diagnoses of these cell lines to MRT by the alteration of INI1 gene (16). Next, the expression of EGFR on two MRT tissues and two MRT cell lines established from these two tumor tissues was determined by immunohistochemistry, immunofluorescence, and Western blot. Furthermore, the antitumor effects of gefitinib on MRT cells in vitro and in vivo are demonstrated, and possible clinical applications of gefitinib for this aggressive and extremely poor prognostic MRT are discussed.

MATERIALS AND METHODS

Tumor Tissues, Cell Lines, and Cell Culture. MRT tissues were obtained from patients AN and NS in 1997 and 1992, respectively. MRT cell lines were established previously from these two patients (designated MP-MRT-AN and KP-
MRT-NS, respectively; refs. 13, 15). MRT was confirmed in the two patients and the two cell lines by clinical, histologic, and ultrastructural examinations. Clinical data on patients AN and NS are given in Table 1. Formalin-fixed (10%) tumor tissues embedded in paraffin were used for the EGFR expressions. The A431 cell line of human epidermoid carcinoma cell line (17) was used as the positive control for the EGFR expression. Cell lines were cultured in RPMI 1640 containing penicillin, streptomycin, and 10% heat-inactivated fetal calf serum at 37°C in a 5% CO₂ incubator.

Reagents. Gefitinib was a kind gift of AstraZeneca (Macclesfield, United Kingdom). Stock solutions were prepared in DMSO and stored at −80°C. EGF was obtained from Life Technologies, Inc. (Carlsbad, CA). Stock solutions were prepared in RPMI 1640 containing 1% fetal calf serum and stored at −80°C.

Reverse Transcription-PCR Analysis. To confirm that our cell lines were MRT cell lines, we checked them for mutations in the hSNF/INI1 (INI1) gene, a tumor suppressor gene for MRT (16). Mutations in this gene are specifically associated with MRT (12). In MP-MRT-AN cells, exons 1–9 of the INI1 gene are deleted.⁴ Two portions of INI1 were amplified by reverse transcription-PCR (RT-PCR), INI1CD1 (the portion corresponding to exons 1–6 of the INI1 gene) and INI1CD2 (the portion corresponding to exons 5–9). RT-PCR was performed according to our institution’s protocol (18). In brief, total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA) from cultured cells and lymphocytes as positive control. Single-stranded cDNA synthesis from a 20-μg sample of template RNA with Oligo-dT (12–18) was performed using reverse transcriptase isolated from Avian Myeloblastosis Virus (20 units, Promega, Madison, WI).

RT-PCR amplification of cDNA, with corresponding 2 μg RNA, were performed in a 50-μg reaction mixture containing 10 mmol/L Tris, 50 mmol/L KCl, 200 μmol/L each of 4 deoxyribonucleotide 5’ triphosphates, 0.2 μmol/L each of primers, and 1.25 units of Taq polymerase (Takara Shuzo, Otsu, Japan). Two pairs of primers were designed to amplify the INI1 cDNA according to the procedure of Biegel et al. (19): INI1CD1.forward, 5’-CTG AGC AAG ACC TTC GGG CAG-3’, and INI1CD1.reverse, 5’-GAT GGC TGG CAC AAA CGT CAG-3’; and INI1CD2.forward, 5’-AGA TCG ATG GGC AGA AGC TGC-3’, and INI1CD2.reverse, 5’-TGG AAT GTG TAC CGG GAA GGG-3’. The primer sequences of β-actin were 5’-GTC GGG CGC CCC ACC CAC CA-3’ and 5’-CTC CTT ATT GTC ACG CAC GAT TTC-3’ (13). The PCR conditions were as follows: initial denaturation at 94°C for 4 minutes, followed by 30 cycles of 94°C for 25 seconds, 66°C for 1 minute, and 72°C for 2 minutes and final extension at 72°C for 10 minutes. Analysis of PCR products was performed by 2% agarose gel electrophoresis.

Immunofluorescence. MRT cells were cultured on coverslips, fixed for 20 minutes in methanol, rehydrated in PBS, incubated with EGFR monoclonal antibody (1:80; Oncogene, Boston, MA) for 40 min, incubated with fluorescein isothiocyanate-conjugated antimouse IgG (1:80; Cappel Products, Aurora, OH) for 40 min, and observed with an Olympus BX52 immunofluorescence microscope equipped with a CCD camera (DP70, Olympus, Tokyo, Japan) as reported previously (13).

Histochemistry and Immunohistochemistry. Five-μm sections made from paraffin-embedded samples were stained with hematoxylin and eosin. Immunohistochemistry was performed on a section made serially to an hematoxylin and eosin-stained section. Sections were immunostained by a two-step procedure with a dextran polymer conjugate (EnVision+ system, DAKO, Glostrup, Denmark; ref. 20) following the manufacturer’s instructions. Briefly, after deparaffinization and rehydration, endogenous peroxidase activity was blocked incubating the section in 0.03% hydrogen peroxide solution for 5 min. Then, all sections were incubated with Proteinase K solution (DAKO) for 5 minutes. The sections were incubated with anti-EGFR monoclonal antibody (1:200; DAKO) at room temperature for 30 min. Subsequently, slides were incubated with peroxidase-labeled polymer conjugated to goat antimouse immunoglobulin at room temperature for 30 min. The sections were incubated in 3,3’-diaminobenzidine tetrahydrochloride for 10 min to stain the antigen brown and then counterstained with hematoxylin.

Cell Growth Assay. Cells (5 × 10⁴) were plated in 24-well cell plates. After 24 hours, cells were treated with gefitinib or DMSO for an additional 96 hours. The cells were lysed under hypotonic conditions as described previously (21), and nuclei were counted every 24 hours with a Coulter counter (22). All of the experiments were conducted on three separate occasions for each cell line.

Immunoblot Analyses. Cells were washed with Tris-buffered saline [10 mmol/L Tris-HCL (pH 7.6) and 150 mmol/L NaCl], scraped in NP40 buffer [10 mmol/L Tris (pH 8.0), 150 mmol/L NaCl, 1% NP40, 1 mmol/L sodium metavanadate, 3.3 μmol/L pepstatin, 2 μmol/L bestatin, 10 μmol/L leupeptin, 5.25 μg aprotinin, and 1 mmol/L phenylmethylsulfonyl fluoride], and incubated at 4°C for 10 minutes. Lysates were cleared by centrifugation at 4°C for 10 minutes at 20,000 × g. The protein concentrations of the supernatants were determined using the Lowry assay (Bio-Rad, Hercules, CA) and adjusted to equal concentrations (13).

Lysates were suspended in 2 × SDS sample buffer, boiled for 3 minutes, separated by SDS-PAGE (using equal amounts of protein in each lane), transferred to an Immobilon-P membrane, immunoblotted using anti-EGFR monoclonal antibody (1: 2500, Table 1: Clinical histories of MRT patients from whom MP-MRT-AN and KP-MRT-NS cell lines were established

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>MP-MRT-AN</th>
<th>KP-MRT-NS</th>
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<tbody>
<tr>
<td>Age at diagnosis</td>
<td>3 months</td>
<td>2 months</td>
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<tr>
<td>Sex</td>
<td>Female</td>
<td>Female</td>
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<tr>
<td>Site of primary tumor</td>
<td>Liver</td>
<td>Left kidney</td>
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<td>Source of tissue</td>
<td>Peritoneal fluid</td>
<td>Ascitic fluid</td>
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<tr>
<td>Sampled age</td>
<td>10 months</td>
<td>9 months</td>
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<tr>
<td>Outcome</td>
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⁴ Unpublished observations.

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Transduction Laboratories), β-actin antibody (1: 2000, Sigma), anti-c-jun NH2-terminal kinase (JNK) antibody (1:1000, Cell Signaling, Beverly, MA), anti-phosphorylated JNK antibody (1:1000, Cell Signaling), and anti-phosphorylated AKT (1:1000, Cell Signaling). Antibody binding was detected with an enhanced chemiluminescence detection system (Amersham; ref. 23).

The levels of EGFR protein were quantified with NIH Image Software 1.55 (NIH, Bethesda, MD) and were normalized against the levels of β-actin protein. Values are the mean ± SE of results from three separate experiments.

**Immunoprecipitation.** Lysates were prepared as described above, incubated with the appropriate amount of anti-EGFR monoclonal antibody (Transduction Laboratories, Lexington, KY) and 30 µL of protein A/G-plus agarose (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight, washed three times with lysis buffer, boiled in 1 × SDS sample buffer for 3 minutes, and separated by SDS-PAGE. The bands were transferred to an Immobilon-P membrane (Millipore). The membrane was blocked for 2 hours in Tris-buffered saline containing 0.5% Tween 20 with 1% bovine serum albumin, incubated with antiphosphotyrosine antibody (PY20, Transduction Laboratories) or anti-EGFR monoclonal antibody (Transduction Laboratories) for 1 hour at room temperature, washed in Tris-buffered saline containing 0.5% Tween 20, and incubated with horseradish peroxidase-conjugated antimouse IgG (1:2000, Amersham) for 1 hour at room temperature. Antibody binding was detected by enhanced chemiluminescence.

**Terminal Deoxynucleotidyl Transferase-Mediated Nick End Labeling Assay.** MRT cells in growth medium were treated with gefitinib or DMSO. After 96 hours, adherent cells were harvested by trypsinization and pooled with floating cells. A terminal deoxynucleotidyl transferase-mediated nick end labeling assay was performed using a MEBSTAIN apoptosis kit direct (MBL, Nagoya, Japan). Fluorescein isothiocyanate-positive cells were detected with a FACSCalibur cytomter (Becton Dickinson, Mansfield, MA).

**In vivo Antitumor Activity.** Female athymic mice (BALB/c, nu/nu), approximately 4–6 weeks of age, were purchased from CLEA Japan Inc. (Tokyo, Japan). This experimental procedure was approved by the Committee for Animal Research, Kyoto Prefectural University of Medicine, Graduate School of Medicine. Mice were acclimatized for 1 week before being injected with MRT cells. MRT cells, which had been resuspended in 200 µL of PBS, were subcutaneously injected into the dorsal area of athymic mice. From 1 day or 7 days after inoculation, 8 mice per group were treated for a total of 3 or 4 weeks (5 days/week) with: (1) gefitinib in 1% Tween 80, administered at 150 mg/kg/day by oral gavage, or (2) vehicles in 1% Tween 80 by oral gavage. Tumor diameters were serially measured with calipers, and tumor volumes were calculated by the formula π/6 × larger diameter × (smaller diameter)² (9).

An in situ terminal deoxynucleotidyl transferase-mediated nick end labeling assay was performed as described (24). Briefly, the tumor was diced into 5 × 5-mm sections and was fixed in 4% paraformaldehyde for 48 hours, and then embedded in paraffin. The sections were deparaffinized, rehydrated, incubated in 0.03% hydrogen peroxide solution for 5 minutes to block endogenous peroxidase activity, incubated with Proteinase K solution (DAKO) for 5 minutes, incubated with reaction regent including Terminal Transferase, Biotin 16-dUTP, and terminal deoxynucleotidyl transferase-mediated nick end labeling Dilution Buffer (Roche, Indianapolis, IN) for 60 minutes at 37°C, incubated in the presence of peroxidase-conjugated streptavidin (DAKO) for 30 minutes at room temperature, incubated in 3,3′-diaminobenzidine for 10 minutes to stain the antigen brown, and then counterstained with methyl green.

**Statistical Analysis.** Values are expressed as the mean ± SE. The results of the apoptosis assay were compared with an ANOVA and a post-hoc Fisher’s projected least significant difference test to correct for multiple comparisons. The results of the in vivo studies were examined using Student’s t test. All of the Ps represent two-sided tests of statistical significance.

**RESULTS**

**Confirmation That Cell Lines Are MRT Cell Lines.** RT-PCR failed to detect either INI1CD1 (exons 1–6 of the INI1 gene) or INI1CD2 (exons 5–9 of the INI1 gene) in the MP-MRT-AN cell line, and in the KP-MRT-NS cell line, it failed to detect INICD1 and detected a shortened form of INICD2 (Fig. 1). These results indicate that the INI1 gene has a deletion and confirmed that MP-MRT-AN and KP-MRT-NS are MRT cell lines, because mutations in INI1 gene are specifically associated with MRT (12).

**Expression of EGFR in MRT Clinical Tissues and Cell Lines.** We examined EGFR expression in two MRT tissues (AN and NS) from which MP-MRT-AN and KP-MRT-NS cell lines were established. EGFR immunoreactivity was diffuse in AN tissue or nodular in NS tissue, respectively (Fig. 2, A–D). Subsequently, the EGFR expression in two MRT cell lines was examined by immunofluorescence and Western blot. Surface EGFR expression was detected on two MRT cell lines by fluorescence microscopy (Fig. 2E). Over 90% of MP-MRT-AN cells and ~40% of KP-MRT-NS cells were strongly stained.

![Fig. 1 RT-PCR analysis of INI1 gene expression in MRT cell lines.](image-url)
Expression of EGFR in two MRT cell lines was quantitatively determined by Western blot. EGFR expression in both MRT cell lines was lower than that in the A431 cell lines. EGFR expression in the MP-MRT-AN cell line was 1.54 ± 0.036 times more than that in the KP-MRT-NS cell line (Fig. 2F).

Inhibition of EGFR Phosphorylation by Gefitinib in MRT Cell Lines. Because the two MRT cell lines express surface EGFR, the ability of gefitinib to inhibit EGFR phosphorylation was examined. Baseline phosphorylation of EGFR was absent in both MRT cell lines cultured in serum-free medium (Fig. 3A). However, treatment with EGF (50 ng/mL) for 5 minutes induced strong tyrosine phosphorylation of EGFR in both MRT cell lines (Fig. 3A). This effect was decreased in a dose-dependent manner by pretreatment with gefitinib (0.01–20 μmol/L) before the addition of EGF (Fig. 3A). Inhibition was clearly evident with the concentration of gefitinib as low as 0.01 μmol/L. However, the total amount of EGFR was not changed by EGF, gefitinib, and/or DMSO.

Inhibition of MRT Cell Growth by Gefitinib In vitro. Effects of gefitinib on the proliferation of two MRT cell lines were determined in vitro. When the two MRT cell lines were treated with gefitinib at concentrations from 0.1 to 100 μmol/L during the linear growth phase, the proliferation of each MRT cell line was inhibited in a dose-dependent manner (Fig. 3, B and C). In this assay system, the IC50 value of the antiproliferation effect of gefitinib was 10 μmol/L in MP-MRT-AN and was 12 μmol/L in...
as shown in Fig. 3, the growth of MP-MRT-AN and KP-MRT-NS cells was inhibited after 48 hours of gefitinib treatment (10 or 20 μmol/L).

Next, we attempted to determine whether the inhibition of cell growth by gefitinib was associated with either apoptosis or cytostasis. Tumor cells were harvested every 24 hours after gefitinib treatment (5, 10, or 20 μmol/L), and apoptotic cells were examined by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. The results showed that the inhibition of cell growth by gefitinib was accompanied by an increase in the percentage of apoptotic cells. However, the percentage of apoptotic cells was not significantly different between the gefitinib-treated and control groups. Therefore, the inhibition of cell growth by gefitinib was primarily due to cytostasis, rather than apoptosis.

Fig. 3 Effect of gefitinib on two MRT cell lines. A, inhibition of EGFR phosphorylation was clearly evident with the concentration of gefitinib as low as 0.01 μmol/L. Cells were placed in serum-free medium for 24 hours and then treated with the indicated concentrations of gefitinib for 30 minutes. Control cells received drug vehicle: DMSO, final concentration 0.2%. Cells were then stimulated (+) or not (−) with EGF for 5 minutes, lysed, and immunoprecipitated with anti-EGFR antibody. Immunoprecipitates were eluted, separated on a 7.5% SDS-polyacrylamide gel, and probed with anti-phosphotyrosine antibody (top). The blot was then stripped and reprobed with anti-EGFR antibody (bottom). Blots are representative of three separate experiments. IP, immunoprecipitation; p-Tyr, phosphorylase. B and C, inhibition of tumor growth of MRT cell lines by gefitinib. MRT cells were seeded and allowed to attach for 24 hours, and cultured in medium containing serial dilutions of gefitinib for 96 hours in triplicate cultures. Growth was assessed by lysing the cells and counting nuclei. Values are the mean of results from three wells from one of three similar experiments; bars, ±SE. IC50 values are given in Results. D and E, cell growth curves of two MRT cell lines. MRT cells were cultured as described above. Cells were harvested every 24 hours, and nuclei were counted. Values are the mean of results from three wells from one of three similar experiments; bars, ±SE. ●, Control; ■, gefitinib10 μM; ▲, gefitinib20 μM.
transferase-mediated nick end labeling assay (Fig. 4A). Apoptosis was observed after 48 hours of gefitinib treatment at concentrations more than the IC₅₀ values (data not shown), and the maximum level of apoptosis was observed after 96 hours of gefitinib treatment. The effect of gefitinib on the number of apoptotic cells after 96 hours of treatment was dose dependent. Induction of apoptosis was significantly greater at 20 μmol/L gefitinib (MP-MRT-AN, 42.9% and KP-MRT-NS, 47.2%) than at 5 (MP-MRT-AN, 4.5% and KP-MRT-NS, 5.4%) and 10 μmol/L (MP-MRT-AN, 17.9% and KP-MRT-NS, 10.2%; P < 0.05, multiple comparisons using Fisher’s projected least significant difference test; Fig. 4B). Therefore, growth inhibition of the MP-MRT-AN and KP-MRT-NS cell lines induced by treatment with gefitinib at concentrations equal to or lower than IC₅₀ (5 and 10 μmol/L) was due to cytostasis, whereas growth inhibition by gefitinib at a concentration greater than IC₅₀ (20 μmol/L) was due to apoptosis.

**Phosphorylation of JNK and AKT by Gefitinib in MRT Cell Lines.** To determine whether gefitinib treatment induces apoptosis, we examined the effects of gefitinib on the phosphorylation status of signal transduction mediators that are associ-
Effects of Gefitinib on Malignant Rhabdoid Tumor

Effects of gefitinib on malignant rhabdoid tumor with MP-MRT-AN cells (2633/vehicle-treated group by day 31, in both the mice xenografted gefitinib-treated group was significantly smaller than that in the drugs of administration (Fig. 5). Mean tumor volume in the tumors in the gefitinib-treated group grew slowly throughout the course of administration (Fig. 5). Although tumors in vehicle-treated mice grew exponentially, xenografted with KP-MRT-NS cells had single palpable tumors. 8 mice xenografted with MP-MRT-AN cells and 8 of 8 mice started 1 day after injection. One week after the injection, 5 of 8 mice xenografted with KP-MRT-NS cells had single palpable tumors.

This is the first report of the expression of EGFR in two clinical MRT tissues by immunohistochemical examination (Fig. 2, A–D). Expression of EGFR was also demonstrated in two MRT cell lines derived from these two MRT tissues by immunohistochemical examination (Fig. 2E) and Western blot (Fig. 2F). These cell lines have an altered INI-1 gene (Fig. 1). Alterations in this gene are restricted to MRT in pediatric tumors (12). Our obtained results are consistent with the recent detection of

Fig. 5 Inhibition of tumor growth of MRT cell lines (MP-MRT-AN and KP-MRT-NS) in athymic mice by oral administration of gefitinib. Treatment was started 1 day after tumor injection. Once-daily oral gavages of gefitinib at 150 mg/kg (■), or vehicle alone (□) were given for 27 days (5 days/week). Each group contained 7–8 mice. Values are the mean; bars, ±SE. *, P = 0.039; **, P = 0.047 relative to vehicle alone.

DISCUSSION

The objective of this study was to determine the effects of gefitinib, an EGFR-tyrosine kinase inhibitor, on MRT cells and to evaluate the potential of gefitinib as a novel agent for treatment of MRT patients. Our results indicate that targeted inhibition of EGFR-tyrosine kinase is a promising therapeutic strategy for MRT patients that are refractory to other treatments.

Inhibition of Tumor Growth In vivo. The antitumor activity of gefitinib in vivo was examined by oral administration of gefitinib to athymic mice that had been injected s.c. (i.e., xenografted) with 4 × 10⁶ MRT cells. Gefitinib treatment started 1 day after injection. One week after the injection, 5 of 8 mice xenografted with MP-MRT-AN cells and 8 of 8 mice xenografted with KP-MRT-NS cells had single palpable tumors. Although tumors in vehicle-treated mice grew exponentially, tumors in the gefitinib-treated group grew slowly throughout the course of administration (Fig. 5). Mean tumor volume in the gefitinib-treated group was significantly smaller than that in the vehicle-treated group by day 31, in both the mice xenografted with MP-MRT-AN cells [2633 ± 689 mm³ (n = 5) versus 928 ± 65 mm³ (n = 5); P = 0.039] and in the mice xenografted with KP-MRT-NS cells [4360 ± 1374 mm³ (n = 8) versus 1218 ± 458 mm³ (n = 8); P = 0.048]. The experiment was repeated except that gefitinib was started 7 days after tumor injection. Similar results were obtained 24 days after tumor injection, in both the mice xenografted with MP-MRT-AN cells [1116 ± 98 mm³ (n = 7) versus 604 ± 76 mm³ (n = 7); P = 0.0014] and in the mice xenografted with KP-MRT-NS cells [2841 ± 661 mm³ (n = 7) versus 505 ± 150 mm³ (n = 6); P = 0.0086 (Fig. not shown)].

Moreover, we evaluated whether apoptotic cells were induced in the tissues of the xenograft. Terminal deoxynucleotidyl transferase-mediated nick end labeling staining was not significantly different between the vehicle-treated group and the gefitinib-treated group (data not shown). This demonstrated that oral administration of gefitinib inhibited tumor growth in athymic mice due to cytostasis.

activated with apoptosis or cell survival. Two such mediators, JNK1 and JNK2, have been associated with apoptosis when in their activated (phosphorylated) forms. JNK1 and 2 are isoforms of one another with masses of 46 and 55 kDa, respectively (25–28). JNK is a serine/threonine kinase that promotes cell survival when it is in its active form (29). Gefitinib has been shown to inhibit the phosphorylation of AKT (30). Therefore, we evaluate the phosphorylation of JNK and AKT. When incubated in 20 μmol/L gefitinib for 48 hours, both MRT cell lines expressed phosphorylated JNK 1 and 2 (p-JNK1 and 2). However, when incubated in 10 μmol/L gefitinib, MP-MRT-AN expressed p-JNK-2 but not p-JNK-1, whereas KP-MRT-NS did not express either. Thus, activation of JNK2 was persistent at 48 hours after 20 μmol/L gefitinib treatment in both MRT cell lines (Fig. 4C). On the other hand, neither 10 μmol/L nor 20 μmol/L of gefitinib treatment inhibited phosphorylation of AKT in either MRT cell line (data not shown).

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EGFR expression in five MRT cell lines. Moreover, EGFR was found to be phosphorylated by EGF in two MRT cell lines (Fig. 3A). These results prompted us to evaluate the possibility of targeting EGFR for treatment of MRT.

Next, the antitumor effects of gefitinib on two MRT cell lines were evaluated in vitro. Treatment of gefitinib induced a dose-dependent growth inhibition in vitro in two MRT cell lines (Fig. 3, B–E). The growth inhibition induced by gefitinib was due to cytostasis at concentrations near the IC50 values, although it was more due to apoptosis at higher doses (Fig. 3, D and E; Fig. 4A). Our IC50 values of the inhibition of EGFR phosphorylation (Fig. 3A) in MRT cell lines are similar to the IC50 value of other cancer cell lines (0.027–0.033 μmol/L; ref. 7). On the other hand, the gefitinib IC50 values of growth inhibition of head and neck cancer, lung cancer, and breast cancer cell lines were very close to our results under similar experimental conditions (31, 32). Thus, our results are congruent with those reported by other investigators.

We showed that 20 μmol/L gefitinib treatment resulted in persistent phosphorylation of JNK2 (Fig. 4C). JNK activation is induced by sources of stress such as UV light, cytokines, and cytotoxic drugs via mitogen-activated protein kinase kinases 7 and 4 (25). On the other hand, Singh et al. (28) have reported that dephosphorylation of EGFR leads to an increase in phosphorylated JNK1 and 2, and other investigators have reported that persistent activation of JNK1 (26) or activation of JNK2 (29) induce apoptosis. Thus, our results suggest that apoptosis by gefitinib is associated with the JNK pathway via EGFR in MRT cell lines. Moreover, inhibition of AKT activity correlates well with sensitivity to gefitinib (31). However, the importance of AKT inhibition by gefitinib may differ among different tumor types (31). On the basis of our results with MRT cell lines, apoptosis induced by gefitinib is associated with the JNK pathway.

We found that 150 mg/kg gefitinib treatment had no significant apoptotic change in MRT xenograft tissues. The maximum nonlethal dose of gefitinib in mice was reported to be 150 mg/kg (8). Because this is the same concentration at which gefitinib had a cytostatic effect against established MRT xenografts (Fig. 5), the in vivo effect of gefitinib in MRT cells might be limited to the cytostatic effect. Christensen et al. (10) showed that the plasma concentrations of mice given gefitinib orally at a dose of 100 mg/kg for 2 days reached 7 μmol/L. In the present study, the plasma gefitinib concentrations of mice given gefitinib at 150 mg/kg might reach to the levels of 10 μmol/L. Therefore, in accordance with the in vitro results, gefitinib did not induce apoptosis in vivo.

Human tumor xenograft studies have indicated that gefitinib can inhibit the growth of tumors having many EGFR levels (7, 8, 9, 10). In these studies, as in the present study, the effect of gefitinib at the dose of 100–150 mg/kg was cytostasis. Because the inhibition of tumor cell growth by gefitinib in our study was observed in vitro as well as in vivo, our results provide a rationale for evaluating the anticancer activity of gefitinib in patients with MRT.

In a Phase I study, the maximal plasma concentration of gefitinib at the dose of 700 mg/day varied over a wide range (3–7 μmol/L; ref. 33). Gefitinib is metabolized in the liver via the cytochrome P450 3A4 (CYP3A4) pathway (34). The pharmacokinetics of gefitinib in children is not well known. However, because CYP3A4 activity of infants is lower than that of adults (35), it may be possible to achieve plasma gefitinib concentrations of 10 μmol/L and to demonstrate antitumor effects in infant MRT patients.

Oral gefitinib was found to have meaningful antitumor activity in a randomized Phase II trial (13). The response of non-small-cell lung cancers to gefitinib in the above clinical trial was related to adenocarcinoma histology, although high EGFR expression is more common in squamous carcinomas than in adenocarcinomas (11). Thus, the clinical response to gefitinib is not related to the amount of EGFR expression, and the target of gefitinib has not been well clarified.

Indeed, induction of apoptosis and inhibition of growth of MRT cell lines by gefitinib required higher concentrations of gefitinib (20 μmol/L and IC50 = 10–12 μmol/L, respectively) than the inhibition of EGFR phosphorylation (IC50 < 0.1 μmol/L) in a cell-based assay. One possible explanation for the discrepancy in IC50 values between the inhibition of EGFR phosphorylation and cell growth is that the action of gefitinib in MRT may involve inhibition of other tyrosine kinases. Our immunofluorescence studies demonstrated that EGFR expression among the same MRT cells is heterogeneous (Fig. 2E), and EGFR expression levels between the two MRT cell lines are different (Fig. 2F). Thus, the uniformity of gefitinib sensitivity in MRT cell lines raises the possibility that some other tyrosine kinases are involved in gefitinib. The verification of targets of gefitinib and the functions of EGFR in MRT cells may be relevant to the understanding of the molecular basis and pathogenesis of MRT.

MRT is notoriously refractory to present anticancer drugs. Our results indicate that gefitinib may make a positive contribution to the therapy of MRT patients. Additional studies are needed to determine what functions EGFR has in MRT cells and whether MRT patients expressing EGFR tend to have poor prognoses. Moreover, additional clinical trials of MRT patients are needed to determine the efficacy and targets of gefitinib. Because the number of MRT patients is small, such trials may need to be global.

In conclusion, our results demonstrated that gefitinib has antitumor effects on MRT in vitro and in vivo and could be a novel therapeutic agent for improving the prognosis of MRT.

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*In vitro* and *In vivo*

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