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ORIGINAL ARTICLE

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Additive antitumor effects of gefitinib and imatinib on anaplastic thyroid cancer cells

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Abstract *Purpose*: Anaplastic thyroid cancer (ATC) is one of the most aggressive malignancies. Although multidisciplinary treatments have been introduced, patients with this disease rarely survive longer than 1 year. These findings prompted us to investigate the antitumor activity of molecular targeting agents in thyroid cancer cells. *Methods*: Two tyrosine kinase inhibitors, gefitinib and imatinib, were tested in a poorly differentiated thyroid cancer cell line, KTC-1, and two ATC cell lines, KTC-2 and KTC-3. *Results*: All cell lines expressed not only a target molecule of gefitinib, HER1, but also a cognate receptor, HER2. They also expressed target molecules of imatinib, c-ABL and platelet-derived growth factor receptors at various levels. Both agents had modest antitumor activity in these cell lines. Combined treatment with gefitinib and imatinib led to an additional antitumor effect. Each agent induced apoptosis and their combined treatment enhanced apoptosis associated with the down-regulation of antiapoptotic proteins, Bcl-2 and Bcl-xL. Moreover, their combined treatment additionally inhibited the growth of KTC-3 xenografts in nude mice. *Conclusions*: These are the first findings to suggest that both gefitinib and imatinib have antitumor activity against ATC cells and that their combined use has greater activity than either drug alone.

Keywords Anaplastic thyroid cancer · Apoptosis · Bcl-2 · Bcl-xL · Gefitinib · Imatinib

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Introduction

Although the prognosis of patients with differentiated thyroid cancers is generally favorable, anaplastic thyroid cancer (ATC) is one of the most aggressive malignancies in humans. Multidisciplinary treatments, including radiotherapy and cytotoxic chemotherapy, have been conducted to control ATC progression. However, patients with this disease rarely survive longer than 1 year after initial diagnosis [1]. These findings prompted us to investigate novel strategies, such as molecular targeting therapy, to control this lethal malignancy. Recently, two molecular targeting agents, gefitinib and imatinib, have been successfully introduced into clinics for the treatment of patients with solid tumors, non-small cell lung cancer and gastrointestinal stromal tumors, respectively [2, 3].

Aberrant HER1 signaling, such as a high expression of HER1, is associated with the up-regulation of cell proliferation and metastatic potential and shorter survival in patients with solid tumors [4]. Gefitinib is an orally active HER1–tyrosine kinase inhibitor that blocks signal transduction pathways implicated in the proliferation and survival of cancer cells. Preclinical studies indicated that gefitinib inhibits cell cycle progression, invasion, angiogenesis and induces apoptosis in cancer cells expressing HER1. These effects lead to significant growth inhibition in several types of cancers both in vitro and in vivo [5]. Furthermore, clinical trials in patients with non-small cell lung cancer refractory to standard chemotherapy revealed that gefitinib has acceptable tolerability and potent antitumor activity [6]. Gefitinib has been approved for the treatment of patients with advanced non-small cell lung cancer in many countries [2].

Imatinib suppresses signal transduction pathways mediated through c-ABL, c-KIT and platelet-derived growth factor receptors (PDGFRs) [7]. Imatinib is already used to treat patients with chronic myeloid leukemia overexpressing BCR-ABL mutant protein [8]

and gastrointestinal stromal tumors harboring active forms of c-KIT somatic mutations [3]. In addition, preclinical studies have suggested that imatinib has antitumor activity in other malignancies, such as ATC [9], ovarian cancer [10], prostate cancer [11], neuroblastoma [12] and osteosarcoma [13].

Our previous study suggested that the selective suppression of c-ABL activity by imatinib may represent a potential anticancer strategy for ATC [9]. In contrast, another study indicated that imatinib has negligible antineoplastic activity against ATC cells within therapeutically useful concentrations [14]. In addition, two recent reports have suggested that ATC cells consistently overexpress HER1, and gefitinib effectively blocks HER1 activation, inhibits cellular proliferation and induces apoptosis in vitro. An in vivo study also showed that gefitinib has significant antitumor activity against ATC xenografts in nude mice [15, 16]. These findings prompted us to test the hypothesis that combined treatment with imatinib and gefitinib is a more effective treatment for ATC.

Materials and methods

Thyroid cancer cell lines

We recently established three different thyroid cancer cell lines, KTC-1, KTC-2 and KTC-3 and used them in this study. KTC-1 cells were derived from a patient with poorly differentiated papillary thyroid carcinoma. KTC-2 cells were derived from a patient with anaplastic transformation from thyroid papillary carcinoma. KTC-3 cells were derived from a patient with primary ATC. The characteristics of these cell lines have been published elsewhere [17–19]. Summarized characteristics of these thyroid cancer cell lines are shown in Table 1.

Cell culture

Gefitinib and imatinib were kindly provided by AstraZeneca (Macclesfield, UK) and Novartis Pharma (Basel, Switzerland), respectively, dissolved in DMSO and added to the medium at a final concentration of 0.1%.

To investigate the effects of gefitinib and/or imatinib on cell growth, thyroid cancer cells (1×10^5 cells per well) were seeded in 12-well plates (SB Medical, Tokyo, Japan) and grown in RPMI 1640 medium supplemented with 5% FBS at 37°C in a 5% CO_2 atmosphere for 2 days. After washing with phosphate-buffered saline (PBS), the cells were incubated with phenol red-free RPMI 1640 supplemented with 2% dextran-coated charcoal (DCC)-stripped FBS (Hyclone, UT, USA) plus 1–20 μM of gefitinib or imatinib for 3 days. In the combination treatment, the cells were incubated with phenol red-free RPMI 1640 supplemented with 2% DCC-stripped FBS plus gefitinib and/or imatinib (5 μM each) for 3 days. After incubation, the cells were counted with a Coulter counter (Coulter Electronics, Harpenden, UK).

To investigate the effects of gefitinib and/or imatinib on the mRNA and protein expression levels of apoptosis-related factors and cell cycle regulators or on cell cycle progression, semi-confluent cells in six-well plates (SB Medical) were incubated with phenol red-free RPMI 1640 supplemented with 2% DCC-stripped FBS plus gefitinib and/or imatinib (5 μM each) for 3 days. After incubation, the cells were collected and immediately applied to cell cycle analysis or western blot analysis or stored at −80°C for multiplex reverse transcription polymerase chain reaction (RT-PCR) analysis.

Immunocytochemistry

Harvested cells were washed once with cold PBS and centrifuged at room temperature. The cell pellet was fixed with 10% phosphate-buffered formalin overnight and embedded in paraffin. The paraffin sections were dewaxed with xylene, hydrated with PBS, treated with hydrogen peroxide and processed following an immunoperoxidase procedure as described previously [20]. We used a mouse monoclonal anti-c-KIT IgG1 antibody (sc-13508; Santa Cruz Biotechnology, Santa Cruz, CA, USA), a mouse monoclonal anti-c-ABL IgG1 antibody (sc-23; Santa Cruz Biotechnology) and a rabbit polyclonal anti-PDGFR-α IgG antibody (sc-338; Santa Cruz Biotechnology) as primary antibodies. After incubation, the slides were washed in PBS for 15 min, and each secondary antibody (mouse or rabbit) was applied using the LSAB kit (DAKO Corp., Carpinteria, CA, USA) according to the manufacturer's protocol. After washing, color was developed with 5-bromo-4-chloro-3-indoxyl phosphate and nitroblue tetrazolium chloride (DAKO). The slides were then washed in distilled water for 5 min and mounted. Control experiments were performed by substituting normal rabbit or mouse serum for the first antibody.

Table 1 Characteristics of three thyroid cancer cell lines tested in this study

Cell lines	KTC-1	KTC-2	KTC-3
Histological type	Poorly differentiated papillary carcinoma	ATC	ATC
Doubling time (h)	48	36	24
Number of chromosomes	46	109	146
Thyroglobulin expression	Weak	Weak	Weak
In vivo growth	Slow	Regressing	Fast

Immunoblot analysis

Harvested cells were washed once with cold PBS and lysed by 200 µl per well of cold RIPA buffer [50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 1% deoxycholic acid sodium, 0.05% SDS] plus 4 µl per well of protease inhibitor (Sigma Chemical Co., St. Louis, MI, USA). The cell lysate was clarified by centrifugation at 14,000×*g* at 4°C for 10 min. About 20–30 µg of protein was heated in Laemmli gel loading buffer for 5 min at 95°C and subjected to electrophoresis on 12.5% polyacrylamide gel (Bio-Rad, Richmond, CA, USA). The protein was transferred to nitrocellulose membranes (Amersham Life Sciences, Buckinghamshire, UK) and immunoblotted with appropriate primary antibodies: HER1, anti-HER1 goat polyclonal antibody (Santa Cruz Biotechnology); HER2, anti-HER2 mouse monoclonal antibody (Santa Cruz Biotechnology), Bcl-2, anti-Bcl-2 mouse monoclonal antibody (Dako Japan, Tokyo, Japan); Bax, anti-BAX mouse monoclonal antibody (Dako Japan); Bcl-xL, anti-Bcl-xL rabbit polyclonal antibody (BD Biosciences, Franklin Lakes, NJ, USA); p21, anti-p21 mouse monoclonal antibody (Nippon Becton Dickinson, Tokyo, Japan); and p27, anti-p27 mouse monoclonal antibody (Nippon Becton Dickinson). For detection, the blots were incubated with the appropriate secondary antibodies conjugated with horseradish peroxidase (Santa Cruz Biotechnology), and developed using ECL Plus Western Blotting Detection Reagents (Amersham Life Sciences), according to the manufacturer's instructions. Anti-actin monoclonal antibody as an internal control was obtained from Santa Cruz Biotechnology. The protein intensities were normalized to the actin band and quantified by comparing with control cells. The densities were quantified using Quantity One version 2.5 (PDI Inc., Huntington Station, NY, USA). Reproducibility was confirmed in at least two separate experiments. As the positive control for HER1 and HER2, human breast cancer cell lines MDA-MB-231 and KPL-4 were used, respectively [21].

RT-PCR analysis

Total cellular RNA was extracted with a TRIzol RNA extraction kit (GIBCO BRL Life Technologies, Gaithersburg, MD, USA). One microgram of total RNA and 1 µM Oligo(dT) 18 primer in 12.5 µl of diethyl pirocarbonate-treated water were heated to 70°C for 2 min followed by cooling on ice for 1 min. cDNA synthesis was initiated with 200 U of recombinant Molony murine leukemia virus reverse transcriptase (CLONTECH Laboratories, Inc., Palo Alto, CA, USA), and the reaction was allowed to proceed at 42°C for 1 h. The reaction was terminated by heating at 94°C for 5 min. cDNA was dissolved to a final volume of 100 µl by adding 80 µl of diethyl pirocarbonate-treated water and then was frozen at −30°C until use. Oligonucleotide primers for RT-PCR were designed using a published sequence of each target gene and synthesized by the solid-phase triester method. The primers and conditions used and the expected sizes from the reported cDNA sequence are shown in Table 2. When the mRNA expression levels of c-ABL, c-KIT, PDGFR-α, Bcl-2, Bax and BCL-xL were investigated, multiplex PCR was performed to amplify both the internal control gene (*β*-actin) and one of the target genes in a single reaction. The ratios of primer sets between the control gene and the target gene are shown in Table 2. These ratios and the numbers of PCR cycles were determined to amplify both products logarithmically. Each PCR reaction contained 1/100 cDNA, the indicated concentrations of primers of each target gene and the control gene, 200 µM deoxynucleotide triphosphates, 10 mM Tris–HCl (pH 8.8), 1.5–2.5 mM $MgCl_2$, 50 mM KCl, 0.08% Nonident P40 and 1 U of recombinant Thermus aquatics DNA polymerase (MBI Fermentas, Vilnius, Lithuania) in a final volume of 20 µl. After initial denaturation at 94°C for 4 min, various cycles of denaturation (at 94°C for 15 s), annealing (at various temperatures, as shown in Table 2) and extension (at 72°C for 30 s) were performed on a DNA Thermal Cycler 2400 (PC-960G Microplate Gradient Thermal Cycler, Mortlake, Australia). The final extension was

Table 2 Oligonucleotide primers and conditions for multiplex RT-PCR

Target genes	Primer sequences (Forward/reverse)	Annealing temperature (°C)	Primer ratio	No. of PCR cycles[a]	Expected size (bp)
c-KIT	5′-tgacttacgacaggctggtg-3′5′-aaggagtgaacagggtgtgg-3′	59	0.5/2.0	40	327
c-ABL	5′-gcctcagggtctgagtgaag-3′5′-agcagatactcagcggcatt-3′	59	0.6/2.0	40	309
PDGFR-α	5′-gaacgtggtcaacctgttgg-3′5′-aaagttgctcggcaggtcct-3′	59	0.35/2.0	40	413
Bcl-2	5′-acttgtggcccagataggcacccag-3′5′-cgacttcgccgagatgtccagccag-3′	65	0.5/2.0	35	389
Bcl-xL	5′-cccagaaaggatacagctgg-3′5′-gcgatccgactcaccaatac-3′	55	1.0/2.0	35	448
Bax	5′-aagaagctgagcgagtgtctc-3′5′-tgtccagcccatgatggttct-3′	58	1.0/2.0	35	252
p21	5′-actgtgatgcgctaatggc-3′5′-atggtctccctctgctgtcc-3′	60	0.5/2.0	35	233
p27	5′-gaagcactgcagagacatgg-3′5′-tgcgtgtcctcagagttagc-3′	60	1.0/2.0	35	249
β-Actin	5′-tgacggggtcacccacactgtgcccatcta-3′5′-ctagaagcatttgcggtggacgatggaggg-3′	NA	NA	NA	661

NA Not assessable

[a]Numbers of cycles were determined to amplify both products logarithmically and in relatively similar amounts

performed for 5 min. After visualization of the PCR products on 1.2% agarose gel stained with ethidium bromide, gel images were obtained using a FAS-II UV-image analyzer (TOYOBO Co., Ltd, Tokyo, Japan), and the product densities were quantified using Quantity One version 2.5 (PDI Inc.). Reproducibility was confirmed in at least two separate experiments. As the positive control for c-KIT, a human breast cancer cell line, KPL-1, was used.

Cell cycle analysis

To investigate the effect of agents on cell cycle progression, some of the harvested cells were stained with propidium iodide using a CycleTest Plus DNA Reagent kit (Becton Dickinson, San Jose, CA, USA). Flow cytometry was performed with a FACSCaliber flow cytometer (Becton Dickinson), and the DNA histogram was analyzed using CELLQuest version 1.2.2 (Becton Dickinson) as described previously [22]. Reproducibility was confirmed in at least two separate experiments.

Apoptosis assay

Approximately 5×10^5 cells per well were plated into T-25 flasks (Corning Japan, Tokyo, Japan) and cultured in D-MEM supplemented with 5% FBS for 2 days. The cells were then washed twice with PBS and cultured for 3 days in phenol red-free RPMI 1640 supplemented with 2% DCC-stripped FBS plus 5 μM of gefitinib and/or imatinib. Duplicate flasks were trypsinized and harvested. The percentages of apoptotic cells were measured by FACSCaliber flow cytometry (Becton Dickinson) using an in situ cell death detection kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's recommendations, as described previously [21]. Reproducibility was confirmed in at least two separate experiments.

Animal experiments

Semi-confluent KTC-3 cells were trypsinized and harvested, and viable cells were counted in a hemocytometer using trypan blue exclusion. Approximately 2×10^6 KTC-3 cells were injected subcutaneously into both sides of the back of 5-week-old female nude mice (CLEA Japan, Tokyo, Japan). In the gefitinib-treated group, 200 mg/kg gefitinib was administered daily by oral lavage five times a week between days 15 and 22 for 10 days after cell injections. In the imatinib-treated group, 50 mg/kg imatinib was intraperitoneally administered daily five times a week between days 15 and 22 for 10 days after cell injections. In the combination group, both 200 mg/kg gefitinib and 50 mg/kg imatinib were concurrently administered in the same manner. The same volumes of vehicle were administered to the control group in the same manner. Five mice (ten tumors) were treated in each group. Three-dimensional tumor size was measured with calipers twice a week after cell injections, and body weight was measured twice a week. Tumor volume was calculated as the product of the largest diameter, the orthogonal measurement and the tumor depth. All mice were sacrificed by cervical dislocation 4 weeks after cell injections. After measuring tumor weight, tumor samples were fixed with 5% buffered formalin and embedded in paraffin for morphologic analysis. The animal protocols for these experiments were approved by the Animal Care and Use Committee of Kawasaki Medical School.

Statistical analysis

All values are expressed as mean ± SE. ANOVA analysis with StatView computer software (ATMS Co., Tokyo, Japan) was used to compare the differences between two groups. A two-sided P value less than 0.05 was considered statistically significant.

Results

Expression of HER1, HER2, c-ABL, c-KIT and PDGFR-α in thyroid cancer cell lines

All thyroid cancer cell lines tested by western blot analysis expressed both HER1 and HER2. KTC-3 cells expressed a high level of HER1, and KTC-1 and KTC-2 cells expressed a moderate level of HER1. The expression level of HER1 in KTC-1 and KTC-2 cells was comparable with that of MDA-MB-231 human breast cancer cells, which are known to express a high level of HER1 [21]. KTC-2 and KTC-3 cells expressed a higher level of HER2 than KTC-1 cells. The expression level of HER2 in KTC-1 cells was comparable with that of KPL-4 breast cancer cells, which are known to express a high level of HER2 [23] (Fig. 1).

Although KPL-1 human breast cancer cells [24] expressed c-KIT mRNA, no thyroid cancer cell lines expressed a detectable level of c-KIT mRNA. In contrast, both c-ABL and PDGFR-α were constitutively expressed in all thyroid cancer cell lines (Fig. 2). Immunocytochemical analysis also supports these findings (Table 3).

Antitumor effects of imatinib and gefitinib on thyroid cancer cell lines

Imatinib (1–20 μM) dose dependently inhibited the growth of all thyroid cancer cell lines. The 50% growth inhibitory concentrations (IC_{50}s) of imatinib were 10, 8 and 7 μM for KTC-1, KTC-2 and KTC-3 cell lines, respectively (Fig. 3a). Similarly, gefitinib (1–20 μM) dose dependently inhibited the growth of all thyroid

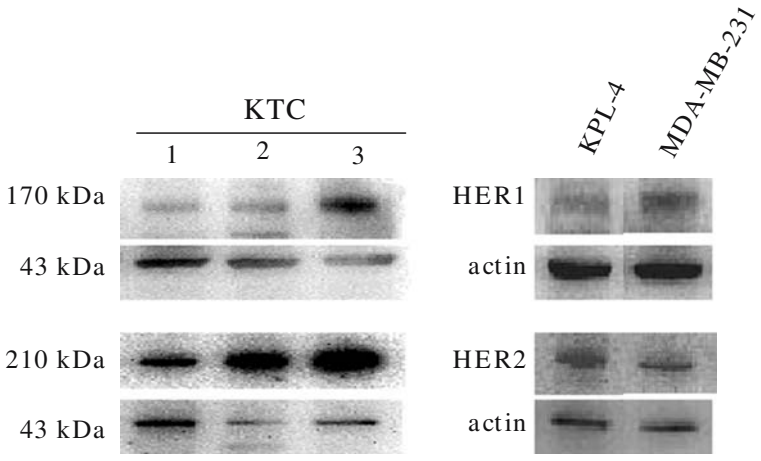


Fig. 1 Western blot analysis on the expression levels of HER1 and HER2 in three thyroid cancer cell lines and two breast cancer cell lines. Actin was used as the internal control. The MDA-MB-231 cell line and KPL-4 cell line are known to express a high expression level of HER1 and HER2, respectively [21]

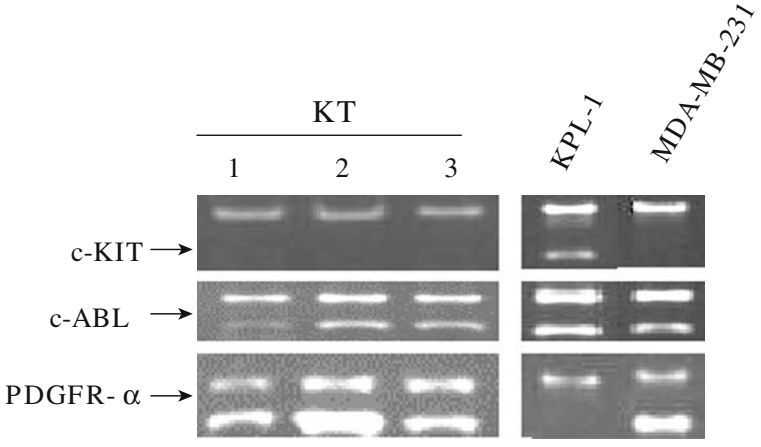


Fig. 2 Multiplex RT-PCR analysis for target molecules of imatinib, c-KIT, c-ABL and PDGFR-α, in three thyroid cancer cell lines and two breast cancer cell lines. The *upper bands* represent β-actin expression as the internal control. The KPL-1 cell line was used as the positive control for c-KIT and MDA-MB-231 as the positive control for PDGFR-α

Table 3 Immunocytochemical analysis of target molecules of imatinib in thyroid cancer cell lines

Cell lines	c-KIT	c-ABL	PDGFR-α
KTC-1	Faint (cytoplasma)	Strong (nucleus)	Strong (cytoplasma)
KTC-2	Not detectable	Moderate (nucleus)	Strong (cytoplasma)
KTC-3	Faint (cytoplasma)	Strong (nucleus)	Strong (cytoplasma)

cancer cell lines. The IC_{50}s of gefitinib were 9, 8 and 15 μM for KTC-1, KTC-2 and KTC-3 cell lines, respectively (Fig. 3b).

Combined treatment with imatinib and gefitinib (5 μM each) was investigated. This combination resulted in an additional antitumor effect on either KTC-2 or KTC-3 cell lines (Fig. 4b, c). However, the addition of gefitinib to imatinib did not increase the antitumor effect in KTC-1 cells (Fig. 4a).

Effects of imatinib and gefitinib on cell cycle progression and apoptosis in KTC-3 cells

To explore the action mechanisms responsible for the additional antitumor effect of imatinib and gefitinib, cell cycle progression analysis was applied to KTC-3 cells. Either imatinib or gefitinib (5 μM each) significantly increased the sub-G1 cell fraction, i.e., the apoptotic fraction, and their combination additionally increased the sub-G1 cell fraction (Fig. 5a). TUNEL analysis also indicated the additional induction of apoptosis by the two agents (Fig. 5b). No significant change in cell cycle progression was observed.

Effects of imatinib and gefitinib on cell cycle regulators and apoptosis-related factors in KTC-3 cells

To further explore the action mechanisms responsible for the additional proapoptotic effect of imatinib and gefitinib, the expression levels of cell cycle regulators, p21 and p27, antiapoptotic factors, Bcl-2 and Bcl-xL, and a proapoptotic factor, Bax, were investigated.

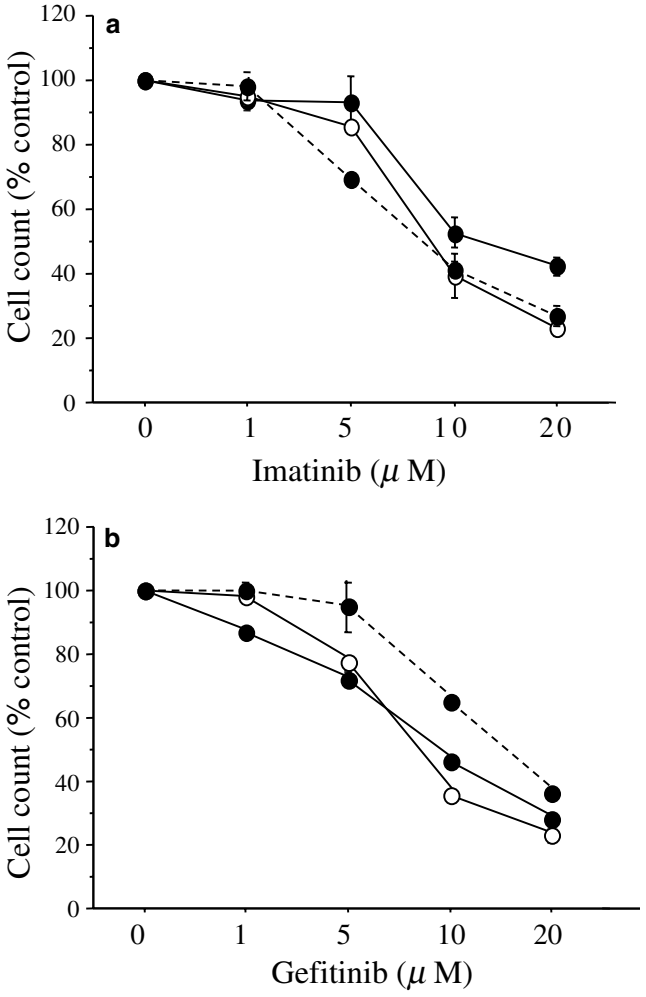


Fig. 3 Dose-dependent antitumor effects of imatinib (**a**) and gefitinib (**b**) on three thyroid cancer cell lines tested. Semi-confluent cells were cultured for 3 days in medium with the vehicle, 1, 5, 10 or 20 μM imatinib or gefitinib. After cell dispersion, the cells were counted using a Coulter counter. Triplicate wells were treated in each experiment. Values represent means ± SE of a representative experiment. *Continuous line with filled circle* KTC-1, *continuous line with open circle* KTC-2 and *broken line with filled circle* KTC-3

Either single treatment with imatinib or combined treatment reduced mRNA and protein expression levels of Bcl-xL in KTC-3 cells (relative expression ratios compared with the respective control were 0.57 ± 0.01 for combined treatment in the RT-PCR analysis and 0.16 ± 0.03 for combined treatment in the western blotting; $P = 0.01$ and $P < 0.01$; Fig. 6a, b). In addition, the combined treatment reduced mRNA and protein expression levels of Bcl-2 in KTC-3 cells (relative expression ratios compared with the respective control were 0.05 ± 0.05 for combined treatment in the RT-PCR analysis and 0.17 ± 0.03 for combined treatment in the western blotting; $P = 0.08$ and $P = 0.06$; Fig. 6a, b). No particular changes in mRNA and protein expression levels of cell cycle regulators, p21 and p27, were observed (Fig. 7).

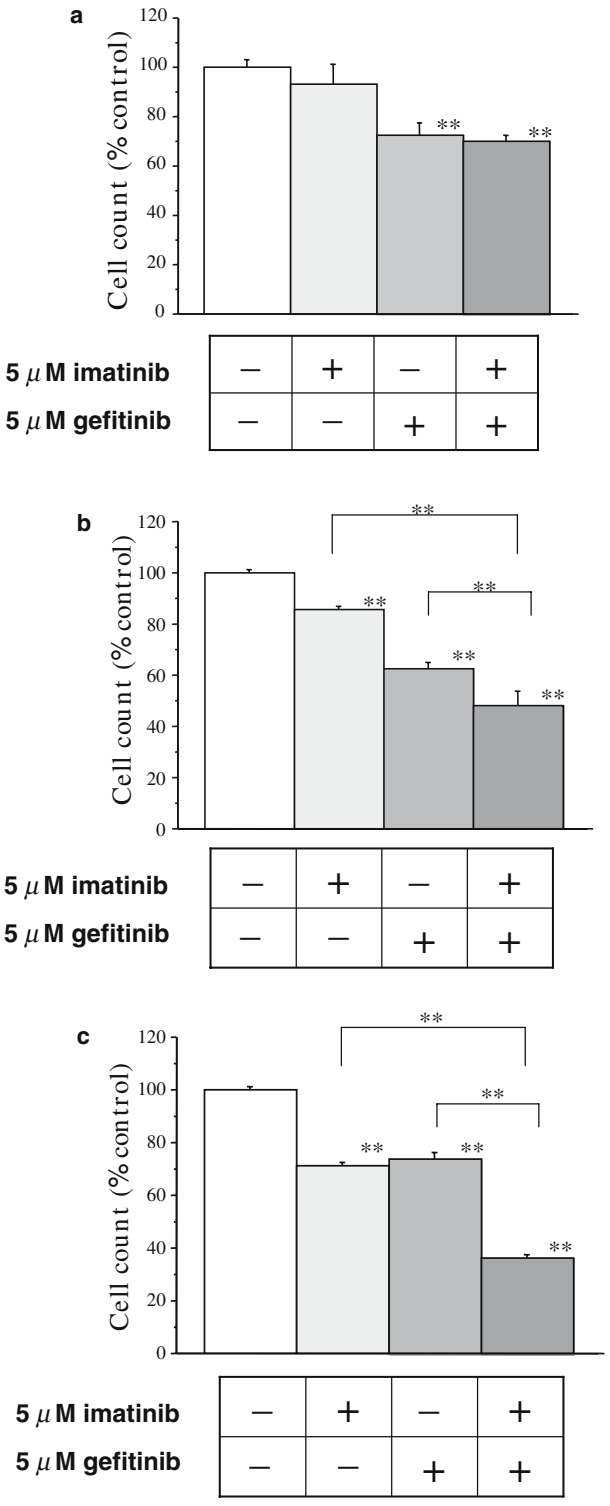
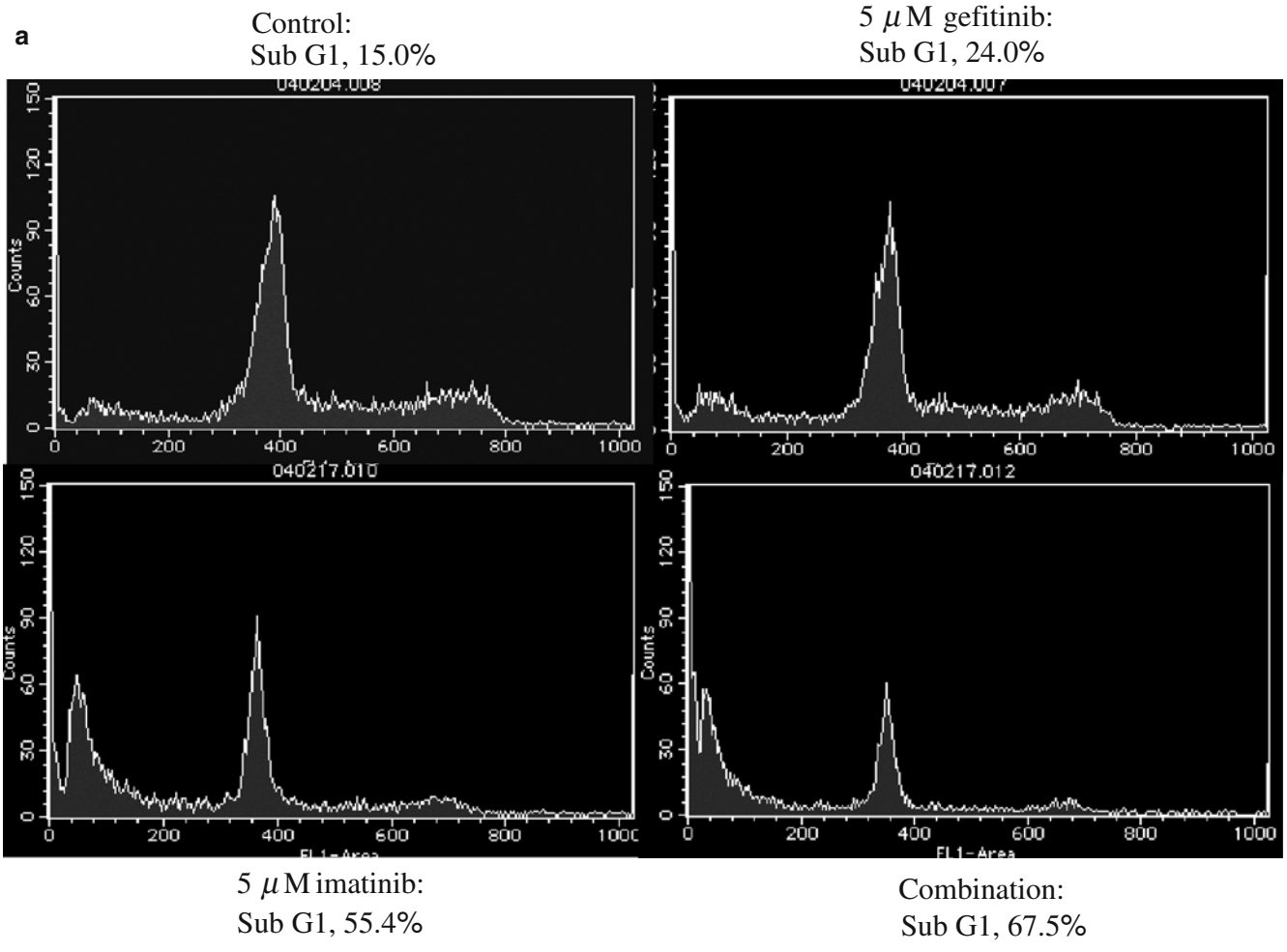


Fig. 4 Combined effects of imatinib and gefitinib on the cell growth of three human thyroid cancer cell lines, KTC-1 (**a**), KTC-2 (**b**) and KTC-3 (**c**). Semi-confluent cells were cultured for 3 days in medium with the vehicle (control), 5 μM imatinib, 5 μM gefitinib or both. After cell dispersion, the cells were counted with a Coulter counter. Triplicate wells were treated in each experiment. Values represent means ± SE of a representative experiment. $^{**}P < 0.01$ compared with the control or compared between a single treatment and combined treatment



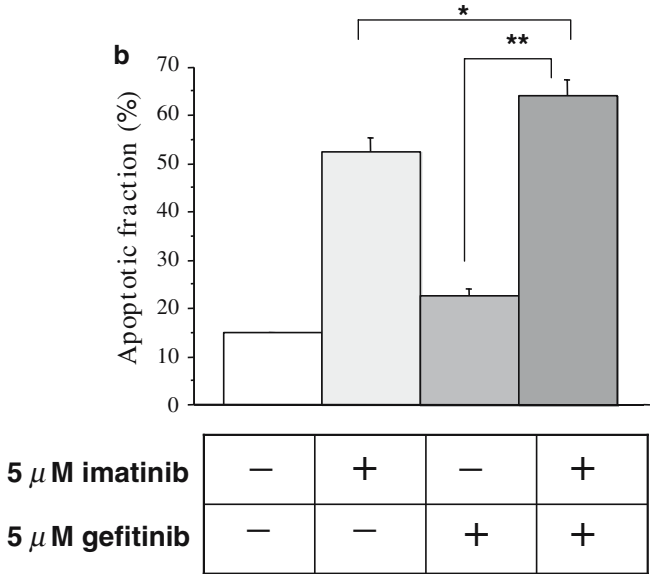


Fig. 5 **a** Flow cytometric analysis of the cell cycle progression in KTC-3 cells treated with imatinib and/or gefitinib. Semi-confluent cells were cultured for 3 days in medium with the vehicle (control), 5 μM imatinib, 5 μM gefitinib or both. The percentages of the sub-G1 fraction in a representative experiment are shown. **b** Analysis of apoptosis in KTC-3 cells treated with imatinib and/or gefitinib. Semi-confluent cells were cultured for 3 days in medium with the vehicle (control), 5 μM imatinib, 5 μM gefitinib or both. The percentages of apoptotic cells were measured with flow cytometry using an in situ cell death detection kit. Values represent means ± SE of a representative experiment. $*P < 0.05$ and $**P < 0.01$ in comparison between combined treatment and respective single treatment

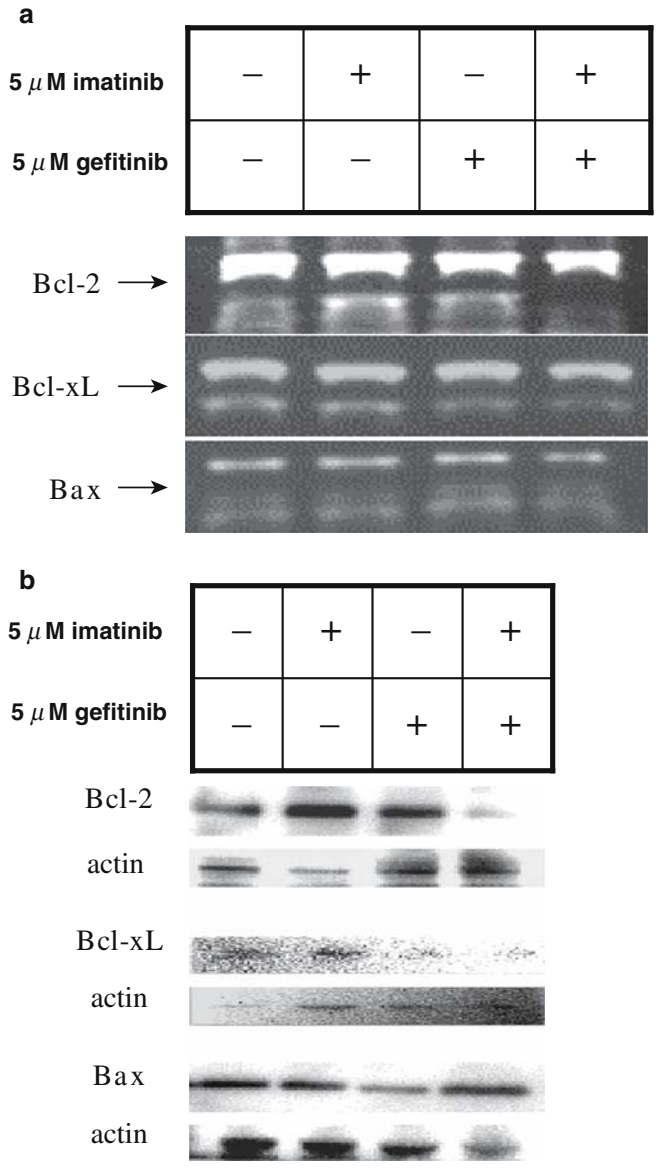


Fig. 6 **a** Multiplex RT-PCR analysis on the expression levels of pro-apoptotic protein BAX and two antiapoptotic proteins, Bcl-2 and Bcl-xL, in KTC-3 cells. Semi-confluent cells were cultured for 3 days in medium with the vehicle (control), 5 μM imatinib, 5 μM gefitinib or both. *Upper bands* represent *β*-actin expression as the internal control. **b** Western blot analysis on the expression levels of a pro-apoptotic protein, BAX, and two antiapoptotic proteins, Bcl-2 and Bcl-xL, in KTC-3 cells. Semi-confluent cells were cultured for 3 days in medium with the vehicle (control), 5 μM imatinib, 5 μM gefitinib or both. Actin was used as the internal control

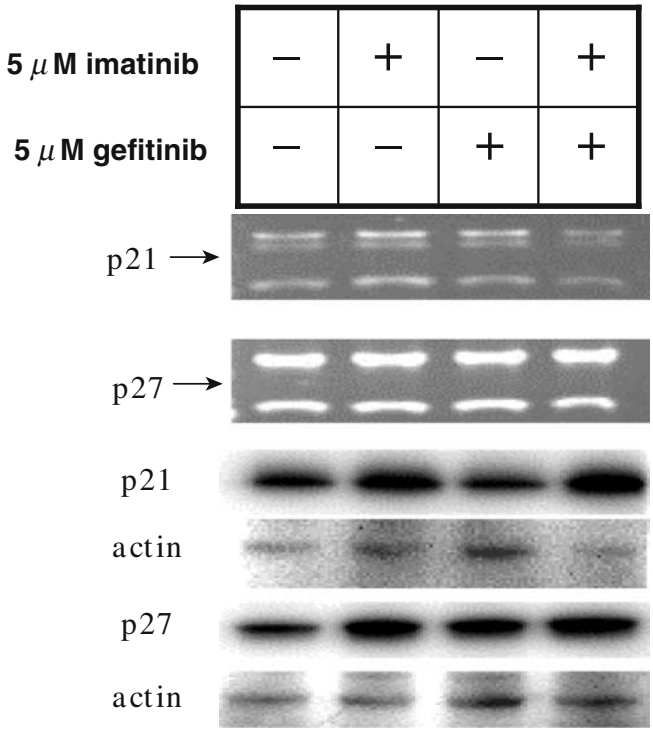


Fig. 7 Analysis of the expression levels of two cell cycle regulators, p21 and p27, by multiplex RT-PCR and western blotting in KTC-3 cells. Semi-confluent cells were cultured for 3 days in medium with the vehicle (control), 5 μM imatinib, 5 μM gefitinib or both. *Upper bands* represent *β*-actin expression as the internal control in RT-PCR analysis. Actin was used as the internal control in western blot analysis

Antitumor effects of imatinib and gefitinib on the KTC-3 xenograft model

Either single treatment with 50 mg/kg of imatinib or 200 mg/kg of gefitinib significantly inhibited the growth of KTC-3 transplanted tumors in nude mice (control of tumor volume at day 14: $55.8 \pm 11.8\%$ for imatinib alone, $P < 0.01$ in comparison with control; $47.4 \pm 12.2\%$ for gefitinib alone, $P < 0.01$). In addition, combined treatment seemed to be more effective than the respective single treatments (control of tumor volume at day 14: $38.1 \pm 5.2\%$, $P < 0.01$; Fig. 8). Tumor weight at day 14 was also significantly lower in each single treatment group and combination group than the control group (data not shown). No significant change in mouse body weight was observed at day 14 (data not shown).

Discussion

Imatinib is a competitive inhibitor of BCR-ABL, c-ABL, c-KIT, PDGFR-α and PDGFR-β tyrosine kinases [7]. It is effective in the treatment of patients with chronic myeloid leukemia expressing the chimeric BCR-ABL gene [8] and with gastrointestinal stromal tumors harboring activation mutations of the c-KIT gene [3]. Preclinical studies also suggest that imatinib is effective against c-ABL and PDGFRs kinase-dependent pathological conditions. The action mechanisms of imatinib have been extensively investigated. Imatinib inhibits the phosphorylation of respective target tyrosine kinases, reduces the activity of downstream effectors such as the RAS pathway, the JAK/STAT pathway and the phosphatidyl inositol-3 kinase pathway, which regulate cellular proliferation, survival and differentiation, and induces cell cycle retardation and/or apoptosis and

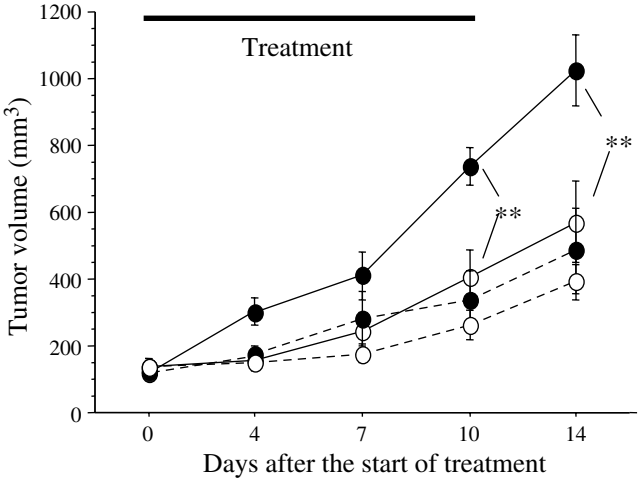


Fig. 8 Antitumor activity of imatinib, gefitinib and their combination in a KTC-3 xenograft model. Athymic nude mice transplanted with KTC-3 cells ($n = 5$ each) were treated with 50 mg/kg imatinib (intraperitoneally), 200 mg/kg gefitinib (orally) or both as described in Materials and methods. Three-dimensional tumor size was measured with calipers twice a week after cell injections. Values represent means ± SE. $^{**}P < 0.01$ compared with the control. *Continuous line with filled circle* control, *continuous line with open circle* imatinib alone, *broken line with filled circle* gefitinib alone and *broken line with open circle* combined treatment

differentiation [7]. It is interesting to note that there are different cellular responses to imatinib depending on the main target tyrosine kinases. Imatinib induces apoptosis in BCR-ABL-positive human leukemia cells in association with the down-regulation of antiapoptotic factors such as Bcl-xL and with the inhibition of Akt and NFκB activities [25]. In contrast, imatinib induces G1-S cell cycle blockade in PDGFR-overexpressing tumor cells in association with the inhibition of Akt activity [10]. In addition, our previous study suggested that imatinib inhibits c-ABL activity, induces S and G2/M cell cycle arrest associated with the up-regulation of p21 and p27 and with the down-regulation of cyclins and showed an antiproliferative effect on human ATC cells [9]. In the present study, all three thyroid cancer cell lines expressed c-ABL and PDGFR-α, but little c-KIT expression was observed. Although massive apoptosis was induced by imatinib associated with a significant decrease in BCL-xL expression in KTC-3 cells, no significant change in the expression levels of cell cycle regulators, p21 and p27, was observed (Figs. 5, 6, 7).

Both HER1 and HER2 were expressed in all three thyroid cancer cell lines tested in the present study. All cell lines were derived from different patients with poorly differentiated thyroid cancer or ATC associated with distant metastases. It has been suggested that HER1 expression is higher in ATC than differentiated papillary thyroid cancer or normal thyroid tissue, and that HER1 expression is increasing, associated with malignant progression in thyroid cancer [26, 27]. Recently, Schiff et al. reported for the first time that a HER1 tyrosine kinase inhibitor, gefitinib, inhibits the growth of ATC cells. Six ATC cell lines were tested in their study, and five of the six cell lines expressed a high level of HER1. The IC_{50} of gefitinib was 6 μM for one of the ATC cell lines, KAT-4, which expressed a high level of HER1. A modest antitumor effect of gefitinib was also observed in the KAT-4 xenograft model [15]. In the present study, we found that the IC_{50} of gefitinib ranged from 7 to 15 μM in three different thyroid cancer cell lines (Fig. 3b). According to the phase I trials of gefitinib, the mean steady-state trough plasma concentration was 1.1–1.4 μM for a daily oral administration of 400–600 mg and the highest trough concentration was 4.8–5.2 μM for a daily oral administration of 700–1,000 mg [28–30]. The IC_{50}s of gefitinib in Schiff's study and our own was 5–10 times higher than the steady-state plasma concentrations and equivalent to the highest trough concentration in men; however, the IC_{50}s of gefitinib significantly depended on the duration of drug exposure, type of culture medium and analyzing system of cell growth. Our preliminary study showed that the lowest IC_{50} of gefitinib was 2.5 μM in a 5-day-exposure system using the same three cell lines (data not shown).

Recently, it has been recognized that certain somatic mutations in the HER1 gene play an important role in the responsiveness of non-small cell lung cancer to gefitinib [31, 32]. To the best of our knowledge, except for lung cancer, there is only one colorectal cancer reported to harbor such a mutation in the HER1 gene [33]. Schiff et al. [15] reported that their analysis of exons 18, 19 and 21 of the HER1 gene showed no mutations in the six ATC cell lines tested. We have not yet performed mutation analysis of the HER1 gene in the three thyroid cancer cell lines. It has been reported that lung cancer cells harboring such somatic mutations were highly sensitive to gefitinib, and that their IC_{50}s of gefitinib were under 0.1 μM [31, 32]. Therefore, it is unlikely that the three thyroid cancer cell lines tested in this study harbor such somatic mutations in the HER1 gene.

Experimental studies have suggested that gefitinib is effective in cancer cells overexpressing not only HER1 but also HER2 [34, 35]. It is known that ligand-activated HER1 preferentially binds to HER2, leads to heterodimerization and activates intracellular signaling pathways. Gefitinib has been reported to effectively inhibit HER2 phosphorylation in vivo. Therefore, it is possible that cancer cells expressing both HER1 and HER2 are more likely to respond to gefitinib. In this study, the relative expression levels of either HER1 or HER2 were higher in KTC-3 cells than in KTC-1 and KTC-2 cells (Fig. 1) but the IC_{50} of gefitinib in KTC-3 cells was highest among the three cell lines (Fig. 3b). It could be concluded that the expression levels of HER1 and/or HER2 were not related to gefitinib sensitivity in this study. However, recent studies have suggested that, in terms of gefitinib efficacy, there are complicated interactions among HER1 mutations, HER1 gene amplification, Akt activation and HER3 expression [36]. Predictive factors for the response to gefitinib remain to be fully determined.

In conclusion, preclinical studies, including this study, have suggested that specific tyrosine kinase inhibitors, imatinib and gefitinib, inhibit the growth of ATC cells in association with the induction of apoptosis. However, the concentrations of these agents tested in preclinical studies were relatively higher than their clinically relevant concentrations. Therefore, considering the clinical implications, it might be advisable to use concurrent administration. To test this hypothesis, imatinib and gefitinib were concurrently administrated to ATC cells both in vitro and in vivo. Combined treatment with imatinib and gefitinib additionally inhibited the growth of two ATC cell lines, KTC-2 and KTC-3. This treatment also resulted in the additional increase in apoptosis and additional decrease in the expression levels of antiapoptotic factors, Bcl-2 and Bcl-xL. Furthermore, simultaneous treatment with imatinib and gefitinib seemed more active than a respective single treatment in the KTC-3 xenograft model (Fig. 7). These are the first findings to suggest that both gefitinib and imatinib have antitumor activity against ATC cells and that their combined use has greater activity than either drug alone.

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