Antitumor effect of gefitinib on head and neck squamous cell carcinoma enhanced by trastuzumab

NORIO KONDO, YUKARI ISHIGURO, MACHIKO KIMURA, DAISUKE SANO, KYOKO FUJITA, ATSUKO SAKAKIBARA, TAKAHIDE TAGUCHI, GABOR TOTH, HIDEKI MATSUDA and MAMORU TSUKUDA

Department of Biology and Function in Head and Neck, Yokohama City University
Graduate School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 236-0004, Japan

Abstract. The overexpression of EGFR and/or HER-2 is associated with tumor cell resistance to chemotherapy, radiotherapy, disease progression and poor prognosis in patients with a variety of malignant tumors. Treatment combining the EGFR-targeting drug, gefitinib (ZD1839, Iressa) with the HER-2-targeting drug, trastuzumab (Herceptin) has been reported to improve therapeutic efficacy in patients with breast cancer. The purpose of this study was to examine the antitumor effect of this combination on head and neck squamous cell carcinoma (HNSCC) *in vitro*. Cell proliferation was inhibited significantly in two cell lines. Although IC_{50} of gefitinib alone against some cell lines was not reached, it was achieved after being combined with trastuzumab. Furthermore, IC_{50} was lower for the combination than for gefitinib alone in several cell lines. These results suggest that the combination may improve efficacy against HNSCC.

Introduction

The epidermal growth factor receptor (EGFR) belongs to the HER family of receptor tyrosine kinases, which includes EGFR (ErbB1 or HER-1), HER-2 (ErbB2), HER-3 (ErbB3) and HER-4 (ErbB4). The influence of EGFR expression on tumor prognosis and evolution is suggested by earlier studies which show that expression correlates with a high tumor growth rate, poor tumor differentiation and a high metastatic rate in many epithelial tumors (1,2). HER-2 is unique, as none of the known HER family ligands activates HER-2 homodimers (3,4). Instead, HER-2 seems to function primarily as a heterodimerization partner for other HER family members

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Correspondence to: Dr Norio Kondo, Department of Biology and Function in Head and Neck, Yokohama City University Graduate School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 236-0004, Japan
E-mail: t076022c@yokohama-cu.ac.jp

(4,5). HER-2 is expressed in many human malignancies of epithelial origin, including breast, lung, bladder, and head and neck cancer (6,7). Overexpression of HER-2 is associated with a poor prognosis. A high expression of EGFR and/or HER-2 has been associated with tumor cell resistance to chemotherapy and radiotherapy, disease progression and poor prognosis in patients with a variety of cancers (8-12). Thus, EGFR and HER-2 are attractive targets for anticancer treatments.

Molecular targeting drugs including the HER family inhibitors have been developed and tested *in vitro* and in clinical models. Gefitinib (Iressa) is an orally active, synthetic anilinoquinazoline tyrosine kinase inhibitor (TKI) which is highly selective for EGFR and active against human solid tumors (13,14). It blocks signal-transduction pathways implicated in the proliferation and survival of cancer cells, as well as other host-dependent processes that promote cancer growth (15-18). Data from clinical trials indicate that gefitinib has an antitumor effect on head and neck squamous cell carcinoma (HNSCC) (19-22) but that only a minority of HNSCC patients benefit from EGFR-targeted therapies.

Trastuzumab (Herceptin) is a humanized monoclonal antibody at the extracellular domain of HER-2. Clinical trials have clearly demonstrated that trastuzumab has significant activity against metastatic breast cancer (23,24). Although trastuzumab is believed to exert its antitumor effect by inactivating survival signal transduction, the mechanism by which anti-HER-2 antibodies inhibit tumor cell proliferation is not well defined (25). Trastuzumab also has a partial ability to disrupt the heterodimerization of HER-2 with other HER family members, suggesting that it is able to impair signaling through other HER receptors (26). Among the head and neck cancers, only salivary duct carcinoma is reported to be responsive to trastuzumab therapy (27,28), but its effect on HNSCC has yet to be reported. Results of the phase II clinical trial of another HER-2 targeting therapy, lapatinib (a dual inhibitor of EGFR and HER-2 tyrosine kinase activity), suggest it is effective against malignant salivary gland tumors (29).

A previous study suggested that the overexpression of EGFR and HER-2 may act in concert to sustain the autonomous proliferation of breast cancer cells (30-32). Moreover, breast tumors that co-express EGFR and HER-2 have a relatively poor prognosis compared with tumors that express either of these receptors alone (33-35). Targeting

EGFR and HER-2 in cancer treatment is reported to be efficacious for breast, non-small cell lung (NSCLC) and prostate cancer, as well as vulvar squamous cell carcinoma cells (12,36-38). Based on the above findings, we thought it would be noteworthy to target EGFR and HER-2 in the treatment of HNSCC.

The antitumor effect of gefitinib combined with trastuzumab on HNSCC has yet to be reported. The purpose of this study was to determine whether the combination had a significant cytotoxicity against HNSCC cell lines. We examined EGFR and HER-2 expression, and cell growth inhibition in 16 HNSCC cell lines treated with this combination.

Materials and methods

Drugs. Gefitinib (ZD1839, Iressa®) was provided by Astra-Zeneca (Macclesfield, UK). For the antiproliferative assay, stock solution (10 mM) was made in 100% dimethyl sulfoxide (DMSO) and diluted with culture media. Trastuzumab (Herceptin®) was purchased from Chugai Pharmaceutical Co. Ltd. (Tokyo, Japan). For the antiproliferative assay, trastuzumab was dissolved in saline. Dosing preparations of the two agents were prepared on the day of use.

Cell lines and culture conditions. Sixteen human HNSCC cell lines were examined in this study. The origins of these cell lines were the oral floor (YCU-OR891), hypopharynx (YCU-H891), mesopharynx (YCU-M862, KCC-M871 and YCU-M911), larynx (KCC-L871 and YCU-L891), tongue (KCC-T871, KCC-T873, YCU-T891 and YCU-T892), maxillary sinus (KCC-MS871 and YCU-MS861) and metastatic tumors from different tongue carcinomas (KCC-TCM901, KCC-TCM902 and KCC-TCM903). These cell lines were established in the Department of Otolaryngology, Yokohama City University School of Medicine and Research Institute, Kanagawa Cancer Center. All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) (Wako Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with 10% fetal bovine serum (Gibco). These cells were incubated at 37˚C in a moist atmosphere containing 5% CO_2.

Western blot analysis. The 16 human HNSCC cell lines were grown in 100-mm plastic dishes (Falcon, San Jose, CA) for 24 h at a concentration of $5x10^5$ cells/dish. After removal of the media, cells were washed twice with phosphate-buffered saline (PBS) and lysed with lysis buffer [50 mM Tris-HCl pH 8.0, with 150 mM sodium chloride, 1.0% Igepal CA-630 (NP-40), 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate]. After removal of the cell debris by centrifugation, the protein concentration was determined using a BCA protein assay kit (Pierce, Rockford, IL). Equal amounts of protein were separated by SDS-PAGE and then transferred onto PVDF membranes (Millipore, Bedford, MA). Membranes were blocked with blocking reagent-N102 (NOF Corp., Tokyo, Japan), then reacted with (1:2,000 diluted) primary antibodies to EGFR (Santa Cruz Biotechnology, Santa Cruz, CA) and HER-2 (Cell Signaling, Beverly, MA), and a secondary antibody [ECL anti-rabbit IgG, horseradish-peroxidase-linked whole antibody and ECL anti-mouse IgG, horseradish-peroxidase-linked whole antibody (GE Health

Care, UK)]. The reaction was developed using the ECL detection kit (Amersham Biosciences, UK) and exposed to Kodak BioMax XAR film.

In vitro proliferation assays. Cell proliferation assay was performed to assess the effect of treatment on the growth of 16 human HNSCC cell lines. These cells were plated in 96-well flat plates (Sumilon, Sumitomo Bakelite Co. Ltd., Tokyo, Japan) at a concentration of $3x10^3$ cells/well. Plates were incubated for 48 h prior to drug treatment. After 24 h, 48 h or 72 h of exposure to gefitinib alone, or gefitinib and trastuzumab (5 wells of the 96-well plate per experimental condition), the cell proliferation was assayed by incubating with Tetra Color One (Seikagaku Co. Ltd., Tokyo, Japan). Relative growth inhibition was calculated by dividing the number of recovered drug-treated cells by the number of vehicle-treated control cells, and the IC_{50} values were defined as the drug concentrations showing 50% survival.

Statistical analysis. For the statistical analysis of *in vitro* proliferation assays we used the Student's paired t-test. P<0.05 was considered significant.

Results

Western blot analysis of EGFR and HER-2 expression in HNSCC cell lines. To show that sensitivity of HNSCC cells to gefitinib correlated with the expression of EGFR (its target protein), EGFR in HNSCC cell lines was measured by Western blot analysis (Fig. 1A). No correlation was found between the expression of EGFR and the IC_{50} value of gefitinib (Table I). We also measured HER-2 expression in cultured HNSCC cells by Western blot analysis (Fig. 1B) and found no correlation with the IC_{50} value of gefitinib combined with trastuzumab (Table I).

Antiproliferative effects of gefitinib and trastuzumab on HNSCC cells. We determined the antiproliferative effects at different concentrations of gefitinib alone (0-100 µM) and/or combined with trastuzumab at a concentration of 100 µg/ml (0.67 µM) on HNSCC cell lines. The concentration of trastuzumab (100 µg/ml) was selected on the basis of two reports (12,38). As shown in Table I, some cell lines, which were insensitive to gefitinib alone (no IC_{50}), were sensitive to the combination (KCC-T873 after 24 h of drug exposure and YCU-M862 after 24 and 48 h of drug exposure). In four cell lines (KCC-T871 after 72 h of drug exposure, KCC-TCM901 after 48 h of drug exposure, YCU-M862 after 72 h of drug exposure and KCC-M871 after 48 h of drug exposure), the IC_{50} value was lower after exposure to the combination than after exposure to gefitinib alone. Statistically significant cell growth inhibition was observed in two cell lines (YCU-M862 after 24, 48 and 72 h of drug exposure and KCC-T873 after 24 h of drug exposure) (Fig. 2A-D).
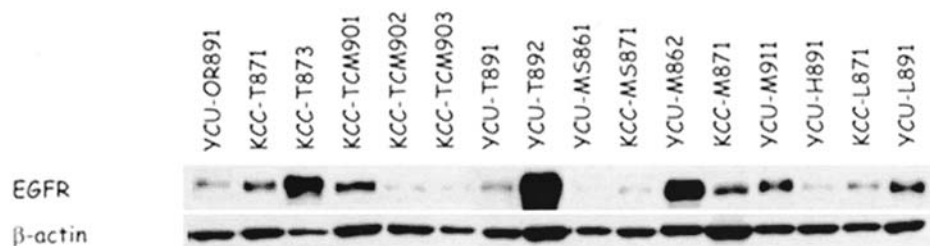
Discussion

EGFR overexpression has been observed in many human cancers. In HNSCC, the overexpression of EGFR has been reported to correlate with reduced survival, increased risk of

Table II. Growth inhibitory effect of the combination treatment of gefitinib and trastuzumab in 16 HNSCC cell lines.

HNSCC cell lines	Primary site	IC$_{50}$ (μM) 24 h		IC$_{50}$ (μM) 48 h		IC$_{50}$ (μM) 72 h	
		G	G + T	G	G + T	G	G + T
KCC-OR891	Oral floor	-	-	13.7	31.0	1.7	4.6
KCC-T871	Tongue	56.9	64.7	46.6	65.2	8.7	**8.2**
KCC-T873	Tongue	-	**51.4**	-	-	84.4	89.2
KCC-TCM901	Lung[a]	59.5	62.7	63.5	**58.9**	64.4	66.6
KCC-TCM902	Lung[a]	97.2	-	60.6	69.5	53.9	61.0
KCC-TCM903	Lung[a]	-	-	28.9	29.2	6.9	8.3
YCU-T891	Tongue	53.2	54.4	35.6	51.8	23.7	31.8
YCU-T892	Tongue	62.4	66.3	52.4	57.0	34.3	38.1
YCU-MS861	Maxillary sinus	-	-	-	-	65.5	76.5
KCC-MS871	Maxillary sinus	83.6	90.0	71.3	73.3	56.5	64.7
YCU-M862	Mesopharynx	-	**58.4**	-	**39.8**	19.0	**8.9**
KCC-M871	Mesopharynx	99.3	-	69.3	**67.5**	51.0	57.5
YCU-M911	Mesopharynx	68.4	71.5	47.4	52.5	52.0	55.5
YCU-H891	Hypopharynx	60.5	64.8	54.9	57.8	55.3	65.4
KCC-L871	Larynx	87.3	95.2	67.0	71.6	52.6	55.8
YCU-L891	Larynx	60.7	66.7	48.8	59.5	14.8	22.7

[a]Metastatic tumors from different tongue carcinomas. G, gefitinib and T, trastuzumab (100 μg/ml). Bold, the IC$_{50}$ values are lower than gefitinib alone and -, the IC$_{50}$ values are not calculated.
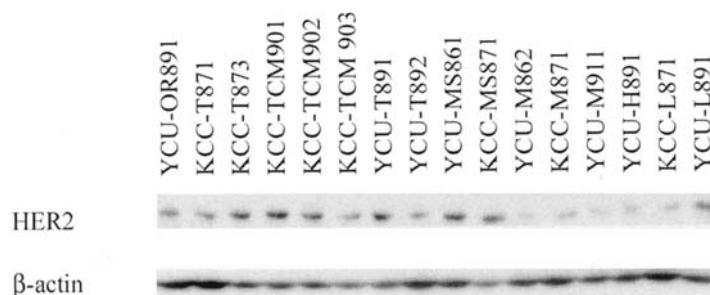
A

B

Figure 1. Expression of EGFR and HER-2 on 16 HNSCC cell lines by Western blot analysis with anti-EGFR and anti-HER-2. ß-actin serves as the control. (A) Expression of EGFR. (B) Expression of HER-2.

recurrence and distant metastasis, and resistance to radio-therapy (1,39-41). Gefitinib has shown an antitumor effect on HNSCC in clinical trials (19-22). However, data from clinical trials indicate that only a minority of HNSCC patients benefit from EGFR-targeted treatment alone. Therefore, another agent combined with gefitinib is needed to boost the clinical effect of gefitinib.

HER-2 is expressed in many human malignancies of epithelial origin, including lung, breast, bladder, and head and neck cancer (6,7). The overexpression of HER-2 is also
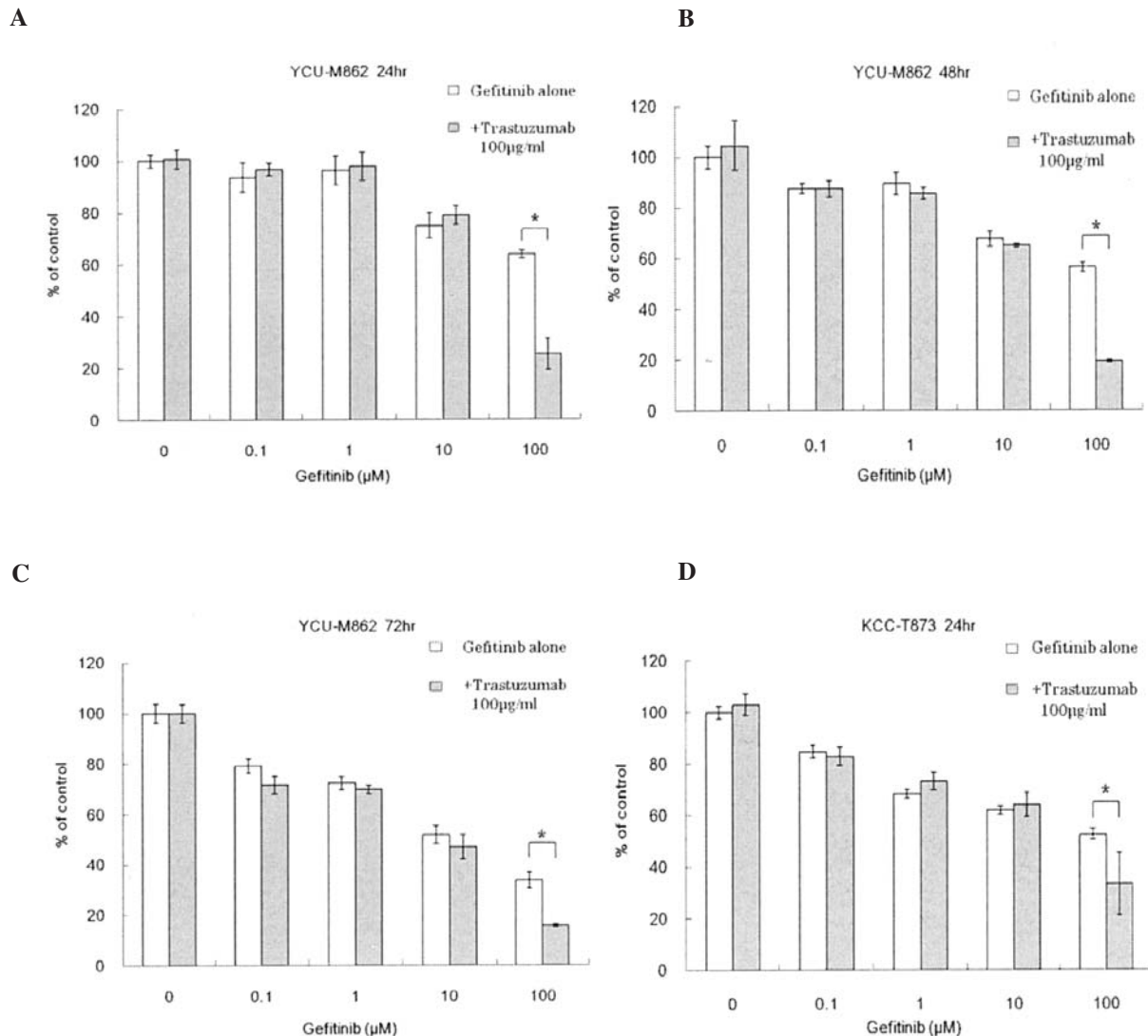
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Figure 2. Growth inhibitory effect of the combination therapy of gefitinib and trastuzumab in YCU-M862 and KCC-T873. (A) YCU-M862 after 24 h of drug exposure. (B) YCU-M862 after 48 h of drug exposure. (C) YCU-M862 after 72 h of drug exposure. (D) KCC-T873 after 24 h of drug exposure. The data presented are mean values (n=5 per group) and the error bars indicate ±SD. *P<0.05 versus gefitinib alone.

associated with poor prognosis in patients with carcinoma. The humanized anti-HER-2 antibody trastuzumab is effective against metastatic breast cancer (23,24). Moreover, several studies have demonstrated a synergistic growth inhibition of breast cancer cells by trastuzumab and gefitinib (30-32). The antitumor effect of this combination on NSCLC and prostate cancer has been reported (36,38). In the treatment of HNSCC, treatment combining gefitinib (targeting EGFR) with pertuzumab (targeting HER-2 heterodimerization) can inhibit tumor growth (4).

Out of 16 HNSCC cell lines, the growth of two (YCU-M862 and KCC-T873) was significantly more inhibited by the combination than by gefitinib alone. In the other 14, growth inhibition by combination was either slightly additive or absent. The IC$_{50}$ values in four cell lines were lower after the combined treatment than with gefitinib alone, while the IC$_{50}$ values in two of these cell lines were not reached after gefitinib alone at the highest concentrations tested.

In two HNSCC cell lines (YCU-M862 and KCC-T873), cell growth was significantly inhibited by the combination. The two lines overexpressed EGFR but not HER-2. In the three cell lines (KCC-T871, KCC-TCM901 and KCC-M871) on which the combination had an additive effect, EGFR but not HER-2 was overexpressed. HER-2 is a dimerization partner for other members of the HER family (42), and EGFR-HER-2 heterodimers are a functionally potent signaling combination (43). Experimental data reported by Christensen and colleagues (44) suggested that it was more difficult to inhibit EGFR phosphorylation in cells expressing particularly high HER-2 levels. A previous study found that HER-2 over-expression reduces the EGFR internalization rate, thereby increasing the fraction of EGFR that is recycled (45). The present data show the relative abundance of EGFR and HER-2, which may play a key role in the final effects on the cell (36).

In the present study, the proliferation of some HNSCC cell lines was inhibited with the combination treatment of

and trastuzumab, but most cell lines showed either ʻffects or no effects at all. Various hypotheses have been proposed to explain the poor sensitivity to this combination. Studies showed that sensitivity to gefitinib is closely associated with somatic mutations in the EGFR catalytic kinase domain at exons 18-21 in NSCLC (46-48). In HNSCC, it has been reported that EGFR mutations are predominant in exon 19 (49,50). The EGFR copy number is associated with poor clinical outcome and sensitivity to gefitinib in NSCLC (51,52) and HNSCC (53). Using direct sequencing of PCR products from exons 18-23 of 16 different HNSCC cell lines, Taguchi *et al* (54) in our department found a heterozygous EGFR mutation with a G→A transition at nucleotide 2607 in exon 20 in nine HNSCC cell lines, which also showed higher sensitivity to gefitinib than cell lines with wild-type EGFR. In terms of HER2, somatic mutation in the HER-2 catalytic domain at exon 20 was associated with survival, invasiveness, tumorigenicity and sensitivity to EGFR TKIs (55).

In HNSCC patients, gefitinib has had an acceptable toxicity profile and promising clinical efficacy in phase I and II trials (19-22). The present study shows that gefitinib may act additively when used concomitantly with trastuzumab, permitting the reduction of the gefitinib dose to reduce its toxicity.

In conclusion, our result demonstrates that gefitinib plus trastuzumab are more effective than gefitinib alone in inhibiting HNSCC cell proliferation. Our data also demonstrate that the level of HER-2 expression is not associated with the efficacy of this combination. The present study provides an experimental basis for the clinical testing of gefitinib plus trastuzumab in HNSCC patients.

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