Afatinib against Esophageal or Head-and-Neck Squamous Cell Carcinoma: Significance of Activating Oncogenic HER4 Mutations in HNSCC

Yu Nakamura1,2, Yosuke Togashi1, Hirokazu Nakahara3,4, Shuta Tomida1, Eri Banno1, Masato Terashima1, Hitotoshi Hayashi5, Marco A. de Velasco1, Kazuko Sakai1, Yoshihiko Fujita1, Takatsugu Okagewa2, Kikuo Nutahara2, Suguru Hamada3, and Kazuto Nishio1

Abstract

The prognosis for patients with advanced esophageal or head-and-neck squamous cell carcinoma (ESCC or HNSCC) remains poor, and the identification of additional oncogenes and their inhibitors is needed. In this study, we evaluated the sensitivities of several ESCC and HNSCC cell lines to HER inhibitors (cetuximab, erlotinib, and afatinib) in vitro and found two cell lines that were hypersensitive to afatinib. Sequence analyses for the afatinib-targeted HER family genes in the two cell lines revealed that one cell line had a previously reported activating EGFR L858R mutation, whereas the other had an HER4 G1109C mutation of unknown function. No amplification of HER family genes was found in either of the two cell lines. The phosphorylation level of HER4 was elevated in the HER4 G1109C mutation-overexpressed HEK293 cell line, and the mutation had a transforming potential and exhibited tumorigenicity in an NIH3T3 cell line, indicating that this HER4 mutation was an activating oncogenic mutation. Afatinib dramatically reduced the phosphorylation level of EGFR or HER4 and induced apoptosis in the two cell lines. In vivo, tumor growth was also dramatically decreased by afatinib. In a database, the frequencies of HER family gene mutations in ESCC or HNSCC ranged from 0% to 5%. In particular, HER4 mutations have been found relatively frequently in HNSCC. Considering the addiction of cancer cells to activating oncogenic EGFR or HER4 mutations for proliferation, HNSCC or ESCC with such oncogenic mutations might be suitable for targeted therapy with afatinib.

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Introduction

Esophageal squamous cell carcinoma (ESCC) and head-and-neck squamous cell carcinoma (HNSCC) are included among the most aggressive cancers. Because of the risk factors commonly associated with the development of ESCC and HNSCC, several studies have revealed that ESCC and HNSCC have similar molecular alterations (1–8). For instance, cyclin D1, p53, EGFR, and c-myc are common important genetic alterations in ESCC and HNSCC carcinogenesis. In addition, it is common to see synchronous and metachronous tumors develop in this region, and the concept of “field cancerization” has become widely accepted (5, 6).

Despite improvements in surgical techniques, chemotherapy, and radiotherapy, the prognosis of ESCC and HNSCC remains very poor. Although recent breakthroughs in molecular biology have assisted in translational research, giving hope for individualized therapy and better disease management, the inhibition of EGFR using cetuximab is, to date, the approved targeted approach for improving survival in patients with HNSCC (9–11). Therefore, further understanding of the molecular biology of ESCC and HNSCC is needed, and the identification of oncogenes with effects similar to those of EGFR mutations or anaplastic lymphoma kinase gene (ALK) rearrangements and upon which cancer cells are dependent is needed (12).

The HER-family of receptor tyrosine kinases consists of four members: EGFR (HER1), HER2, HER3, and HER4. The HER molecule structure has three regions, an extracellular ligand–binding region, and intracellular region with tyrosine kinase activity, and a transmembrane region (13). HER family–related signals are reported to play an important role in modulating cell proliferation, survival, migration, and differentiation. HER family overexpression has been identified in a variety of human cancers including ESCC and HNSCC (14–16). Afatinib, an irreversible HER-family blocker (17, 18), has a remarkable antitumor effect against EGFR-mutated non–small cell lung cancer (NSCLC; refs. 19, 20). In addition, a recent phase III study showed the efficacy of afatinib in patients with recurrent or metastatic HNSCC after the failure of platinum-based therapy (21), and in a phase I study, one of 7 patients with esophageal cancer achieved a...
Materials and Methods

Cell cultures and reagents

The HEK293 (human embryonic kidney cell line), NIH3T3 (mouse fibroblast cell line), human HNSCC cell lines, KYSE30, and KYSE50 (human ESCC cell lines) cell lines were maintained in DMEM with 10% FBS were maintained in DMEM medium (Sigma-Aldrich) supplemented with 10% FBS (GIBCO BRL) in a humidified atmosphere of 5% CO₂ at 37°C. The KYSE170, KYSE180, and KYSE270 cell lines were maintained in a 1:1 mixture of Ham F12 medium (Sigma-Aldrich) and RPMI-1640 medium (Sigma-Aldrich) supplemented with 2% FBS in a humidified atmosphere of 5% CO₂ at 37°C. The KYSE70 cell line was maintained in DMEM with 2% FBS in a humidified atmosphere of 5% CO₂ at 37°C. The KYSE150 cell line was maintained in Ham F12 with 2% FBS in a humidified atmosphere of 5% CO₂ at 37°C. The HEK293 and NIH3T3 cell lines were obtained from the ATCC in 2006. The human ESCC cell lines were obtained in Kyoto University (23–25), and obtained from Japanese Collection of Research Bioresources in 2011. The HNSCC cell lines were also obtained from Japanese Collection of Research Bioresources in 2012. The HEK293, KYSE50, KYSE270, SAS, and OSC-19 cell lines were analyzed using a short tandem repeat (STR) method in August 2015, and the HEK293, KYSE50, KYSE270, SAS, and OSC-19 cell lines were authenticated. Erlotinib and afatinib were purchased from Selleck Chemicals, and cetuximab was purchased from ImClone Systems Incorporated. NRG1, an HER4 ligand, was purchased from R&D Systems.

Cellular growth and growth inhibition assay in vitro

The cellular growth and growth-inhibitory effects of the drugs or siRNA were examined using a 3-4, 5-dimethyl-2H-tetrazolium bromide assay (MTT; Sigma-Aldrich), as described previously (26). The experiment was performed in triplicate.

Sequencing

The PCR reactions were performed using TaKaRa ExTaq (TaKaRa). The PCR products were then directly sequenced using the BigDye Terminator v3.1 Sequencing Kit (Applied Biosystems). The experiment was performed in triplicate.

Copy number assay

The DNA copy numbers of EGFR, HER2, HER3, and HER4 were determined using commercially available and pre-designed TaqMan Copy Number Assays (Applied Biosystems), as described previously (27). The primer IDs used in this study were as follows: EGFR, Hs09974244_cn (intron 14 and exon 15); HER2, Hs05475431_cn (intron 19); HER3, Hs01446234_cn (intron 21 and 22); and HER4, Hs04606638_cn (intron 18). The TERT locus was used for the internal reference copy number. The PCR analysis was performed using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems), and the results were analyzed using CopyCaller software version 2.0 (Applied Biosystems). The experiment was performed in triplicate.

Plasmid construction and transfectants

A pPRE-IRE5-ZsGreen1 vector including a full-length cDNA fragment encoding human HER4 was purchased from Clontech. The HER4 point mutations were amplified using the PrimeSTAR Mutagenesis Basal Kit (TaKaRa). Viral production was performed as previously described (27). The viral vectors and stable viral transfectant cell lines were designated as pPRE-EGFP, pPRE-HER4 WT, pPRE-HER4 G1109C, pPRE-HER4 E317K, HEK293/EGFP, HEK293/HER4 WT, HEK293/HER4 G1109C, HEK293/HER4 E317K, NIH3T3/EGFP, NIH3T3/HER4 WT, NIH3T3/HER4 G1109C, and NIH3T3/HER4 E317K, respectively.

Antibodies

Rabbit antibodies specific for EGFR, phospho-EGFR, HER2, phospho-HER2, HER3, phospho-HER3, HER4, phospho-HER4, AKT, phospho-AKT, ERK1/2, phospho-ERK1/2, poly (ADP-ribose) polymerase (PARP), caspase-3, cleaved PARP, cleaved caspase-3, and β-actin were obtained from Cell Signaling Technology.

Western blot analysis

A western blot analysis was performed as described previously (26). Briefly, subconfluent cells were washed with cold phosphate-buffered saline (PBS) and harvested with Lysis A buffer containing 1% Triton X-100, 20 mmol/L Tris-HCl (pH7.0), 5 mmol/L EDTA, 50 mmol/L sodium chloride, 10 mmol/L sodium pyrophosphate, 50 mmol/L sodium fluoride, 1 mmol/L sodium orthovanadate, and a protease inhibitor mix (Complete, Roche Diagnostics). Whole-cell lysates were separated using SDS-PAGE and were blotted onto a polyvinylidene fluoride membrane. After blocking with 3% BSA in a TBS buffer (pH 8.0) with 0.1% Tween-20, the membrane was probed with the primary antibody. After rinsing twice with TBS buffer, the membrane was incubated with a horseradish peroxidase–conjugated secondary antibody and washed, followed by visualization using an ECL detection system and LAS-4000 (GE Healthcare). When the phosphorylation levels of EGFR, HER4, AKT, and ERK1/2 were examined after inhibitor exposure, the samples were collected 3 hours after stimulation. When the apoptosis-related molecules were examined after inhibitor exposure, the samples were collected 24 hours after stimulation. When the influence of NRG1 was evaluated, the cultured medium was replaced with serum-free medium 8 hours before stimulation and the samples were collected 10 minutes after stimulation.

siRNA transfection

Cells were transfected with siRNA for HER4 and a nonspecific target (scramble) as follows: CCGGAUACUCUCAUUCUUC (siRNA HER4-1) and ACUGAGCCUCUCUCUCUUCAG (siRNA HER4-2) for HER4, and UAUGUACUGUCAUCUC (siRNA scramble-1) and CCGUUUGGCCUCUCGCACCU (siRNA scramble-2) for a scramble of HER4. siRNA transfection was performed using RNAiMAX (Invitrogen) as previously described (28).
Xenograft studies 
Nude mice (6-week-old females; CLEA Japan) were used for the in vivo studies and were cared for in accordance with the recommendations for the Handling of Laboratory Animals for Biomedical Research compiled by the Committee on Safety and Ethical Handling Regulations for Laboratory Animals Experiments, Kinki University. The ethical procedures followed and met the requirements of the United Kingdom Coordinating Committee on Cancer Research guidelines. To evaluate tumorigenicity, a suspension of $1 \times 10^8$ NIH3T3 transfectant cells (in 100 μL of PBS) was subcutaneously inoculated into the right flank of each nude mouse ($n = 5$), and tumor formation was examined after 2 weeks. To evaluate the effects of afatinib, a suspension of $3 \times 10^6$ KYSE270 cells or $1 \times 10^6$ SAS cells (in 50 μL PBS) with 50 μL of Matrigel was subcutaneously inoculated into the right flank of each mouse ($n = 5$). One week after the inoculation, treatment was initiated when the tumors in each group achieved an average volume of approximately 100 to 150 mm$^3$. In the treatment groups, afatinib (5 or 15 mg/kg) was administered by oral gavage daily for 2 weeks; control animals received 0.5% methylcellulose-0.4% polysorbate-80 as a vehicle. The tumor volume was calculated as the length × width$^2$ × 0.5. The tumor formation and volume were assessed twice a week. These methods have been previously described (26, 27).

Database analysis 
To analyze the prevalence of genomic alterations of HER family gene, the database of the cBioPortal for Cancer Genomics (http://www.cbioportal.org/public-portal/) was searched (29, 30) and four datasets were investigated (1, 4, 31, 32). Both the gene mutation and copy-number variation data were analyzed.

Statistical analysis 
Continuous variables were analyzed using the Student t test, and the results were expressed as the average and SD. The quantification of Western blotting was analyzed using ImageJ software (http://imagej.nih.gov/ij/). The statistical analyses were two-tailed and were performed using Microsoft Excel (Microsoft). A P value of less than 0.05 was considered statistically significant.

Results 
KYSE270 and SAS cell lines responded to afatinib 
To evaluate the growth inhibitory effects of cetuximab, erlotinib, or afatinib against seven ESCC and five HNSCC cell lines, we used an MTT assay and calculated the 50% inhibitory concentrations (IC$_{50}$) for each drug (Fig. 1A and B). The IC$_{50}$ values for each drug are summarized in Fig. 1B and Supplementary Table S1. Two cell lines, KYSE270 and SAS, were hypersensitive to afatinib (Fig. 1A and B). In contrast, none of the cell lines were sensitive to cetuximab or erlotinib.

KYSE270 cell line had an EGFR mutation, and SAS cell line had an HER4 mutation 
Next, to elucidate the mechanism of the hypersensitivities of these two cell lines to afatinib, the COSMIC database (Sanger Institute, Hinxton, UK) was searched for afatinib-targeted genes (EGFR, HER2, and HER4), showing that the KYSE270 cell lines harbored an EGFR L861Q mutation and that the SAS cell line harbored an HER4 G1109C mutation, respectively (Supplementary Table S1). As seen in Fig. 2A, the mutations were confirmed using direct sequencing. The EGFR L861Q mutation in the KYSE270 cell line had been previously reported as an activating oncogenic mutation (16, 33, 34), whereas the function of the HER4 G1109C mutation in the SAS cell line was unclear.

The possibility of EGFR amplification as a predictive biomarker for the efficacy of afatinib against HNSCC was demonstrated in a previous study (35). Consequently, a TaqMan copy-number assay was performed to determine the gene amplifications of EGFR, HER2, HER3, and HER4. The copy-number assay revealed that the KYSE30 and OSC-20 cell lines had an EGFR copy number of ≥5 copies (copy number gain), while no copy-number gain of HER2, HER3, or HER4 was observed (Fig. 2B). These cell lines with an EGFR copy number gain were not sensitive to the drugs, including afatinib, and the two cell lines that were hypersensitive to afatinib did not have any gains in HER family gene copy numbers.

Afatinib induced apoptosis in the KYSE270 cell line by inhibiting the EGFR signal 
We examined the phosphorylation levels of EGFR, AKT, and ERK1/2 after afatinib exposure (0, 0.001, and 0.01 μmol/L) in the KYSE270 (EGFR L861Q) and KYSE50 (EGFR wild-type) cell lines. Afatinib significantly decreased the phosphorylation levels of EGFR, AKT, and ERK1/2 in the KYSE270 cell line in a dose-dependent manner, compared with the KYSE50 cell line (Fig. 3A). We then analyzed the apoptosis-related molecules after exposure to afatinib using Western blot analyses. In contrast with the KYSE50 cell line, 24 hours of exposure to afatinib (0.01 μmol/L) greatly increased the levels of cleaved PARP and cleaved caspase-3 in the KYSE270 cell line, but not in the KYSE50 cell line (Fig. 3B). These results suggest that apoptosis is induced in response to afatinib in the KYSE270 cell line carrying the activating EGFR L861Q mutation via the inhibition of the EGFR signal.

In vivo efficacy of afatinib in the KYSE270 cell line 
To further investigate the in vivo efficacy of afatinib against tumor growth, we extended our study to xenografted mice generated by the subcutaneous injection of the KYSE270 cell line. One week after tumor cell implantation, the tumors had achieved an average volume of approximately 100 to 150 mm$^3$. Then, we assigned the xenografted mice to three different groups. In the treatment groups, afatinib (5 or 15 mg/kg) was administered by oral gavage daily for 2 weeks; the control animals received 0.5% methylcellulose-0.4% polysorbate-80. Afatinib significantly inhibited the growth of the KYSE270 tumors in a dose-dependent manner [vehicle: 846.6 ± 362.6 mm$^3$ vs. afatinib 5 mg/kg: 303.2 ± 58.5 mm$^3$ (*, P = 0.028) or vs. afatinib 15 mg/kg: 49.4 ± 27.1 mm$^3$ (**, P = 0.0078); afatinib 5 mg/kg vs. afatinib 15 mg/kg, *P = 0.00017; Fig. 3C].

Her4 G1109C mutation increased the phosphorylation of Her4 
To address the role of the HER4 G1109C mutation, the HER4-overexpressed HEK293 and NIH3T3 cell lines were created using each HER4 expression vector (wild-type, G1109C, or E317K). Because the oncogenic activity of the melanoma-derived HER4 E317K mutation has been previously reported, we used this mutation as a positive control (36). HER4 was strongly phosphorylated in HEK293/HER4 G1109C and NIH3T3/HER4...
G1109C at a level similar to that in HEK293/HER4 E317K and NIH3T3/HER4 E317K, compared with the controls (HER4 wild-type; Fig. 4A). The relative expressions of phosphorylated HER4/HER4 were elevated in the HEK293/HER4 G1109C (1.8) and NIH3T3/HER4 G1109C (2.6) cell lines, compared with those in the controls (HER4 wild-type, 1). The phosphorylation levels of other HER family members (EGFR, HER2, and HER3) in the HEK293/HER4 G1109C cell line were elevated compared with those in the HEK293/HER4 WT cell line, which were similar to the phosphorylation of HER4 (Supplementary Fig. S1). These findings indicate that mutant HER4 can activate other HER family members. When the transfectant HEK293 cell lines were incubated with serum-free medium, the phosphorylation level of HER4 was also elevated in the HEK293/HER4 G1109C cell line, compared with that in the HEK293/HER4 WT cell line (Supplementary Fig. S2). In addition, NRG-1 (50 ng/mL), an HER4 ligand, enhanced the cellular growth and phosphorylation level of HER4 in the HEK293/HER4 G1109C cell line to an extent similar to those in the HEK293/HER4 WT cell line (Supplementary Fig. S2). These findings suggest that this mutation is not associated with the response to the ligand.

HER4 G1109C mutation enhanced cellular growth and had a transformational ability and tumorigenicity

Next, using the transfectant HEK293 cell lines, the cellular growth was compared. The cellular growth of the HEK293/HER4 G1109C cell line was enhanced compared with the controls (Fig. 4B). The enhanced cellular growth was cancelled by afatinib (Fig. 4B). We then investigated the transformational abilities and tumorigenicities of the HER4 mutation using a focus formation assay and a tumorigenicity assay with the NIH3T3 cell line, which demonstrated that the HER4 G1109C mutation had a transformational ability and tumorigenicity (Fig. 4C). No focus or tumor was formed in the controls (EGFP and wild-type). These results indicate that the HER4 G1109C mutation derived from the SAS cell line is an activating oncogenic HER4 mutation with a transformational ability and tumorigenicity.

SAS cell line partially depended on the HER4 signal for its cellular growth

To investigate the role of the HER4 signal in the SAS cell line, the HER4 gene was subjected to knockdown using siRNA. We used two siRNAs for HER4 and a nonspecific target (scramble) to avoid off-target effects (Fig. 4D). The cellular growth of the SAS cell line was significantly reduced by HER4-knockdown, whereas that of the OSC-19 cell line was slightly reduced (Fig. 4D). In addition, the sensitivity to afatinib in the SAS cell line was significantly weakened by HER4-knockdown (Supplementary Fig. S3). These findings indicate that the SAS cell line harboring an activating oncogenic HER4 mutation partially depends on the HER4 signal for its cellular growth.
Afatinib induced apoptosis in the SAS cell line by inhibiting the HER4 signal

We examined whether afatinib also reduced the phosphorylation levels of HER4, AKT, and ERK1/2 and induced apoptosis in the SAS cell line (HER4 G1109C). The OSC-19 cell line (HER4 wild-type) was used as a control. After 3 hours of exposure to afatinib, the phosphorylation levels of HER4, AKT, and ERK1/2 in the SAS cell line were reduced in a dose-dependent manner (0, 0.01, and 0.1 μmol/L; Fig. 5A). In contrast, no significant decrease in the phosphorylation level of HER4, AKT, or ERK1/2 was seen in the OSC-19 cell line (Fig. 5B). We also found that treatment with 0.1 μmol/L afatinib for 24 hours markedly increased the expressions of apoptosis-related molecules (cleaved caspase-3 and cleaved PARP) in the SAS cell line (Fig. 5A). In contrast, these expressions were not changed after treatment with afatinib in the OSC-19 cell line (Fig. 5B). These findings suggest that apoptosis is induced in response to afatinib in the SAS cell line carrying the activating HER4 G1109C mutation through the inhibition of the HER4 signal.

In vivo efficacy of afatinib in the SAS cell line

To further investigate the in vivo efficacy of afatinib against tumor growth, a suspension of SAS cells was subcutaneously inoculated into the flank of each mouse. One week after tumor cell implantation, the tumors had achieved an average volume of approximately 100 to 150 mm³ and the treatment was initiated similarly to the KYSE270 cell line. The tumors from the SAS cell line were reduced by treatment with afatinib in a dose-dependent manner (vehicle: 1,433.3 ± 200.2 mm³ vs. afatinib 5 mg/kg: 931.0 ± 265.5 mm³, *P = 0.011) or vs. afatinib 15 mg/kg: 507.5 ± 173.8 mm³, *P < 0.0001). afatinib 5 mg/kg vs. afatinib 15 mg/kg, *P = 0.021; Fig. 5C).

Frequency of HER family gene alterations in ESCC and HNSCC

To analyze the prevalence of HER family gene alterations, the database of the cBioPortal for Cancer Genomics (http://www.cbioportal.org/public-portal/) was searched (29, 30) and four datasets were investigated in the database (1, 4, 31, 32). Although several data items were not available, the analyzed data are summarized in Table 1. In ESCC, EGFR amplification was frequently found (about 25%), whereas the frequency of EGFR mutation seemed to be very low. Similarly, the frequency of EGFR amplification was relatively high (about 10%) in HNSCC. In contrast, HER4 mutations were relatively frequent among HNSCC (~5%). In HNSCC, the biologic functions of all the identified HER4 mutations remain unknown, and there does not seem to be any recurrence or hotspot (Supplementary Fig. S4). Some of the identified mutations were located close to previously reported activating HER4 mutations in melanoma, which are located in various domains, including a growth factor receptor, receptor L, and tyrosine kinase domains (36). One mutation (N1062H) was located close to this cBioPortal experiment was performed in triplicate. The KYSE50 and OSC-20 cell lines had an EGFR copy number of ≥5 copies (copy number gain), whereas no copy number gain of HER2, HER3, or HER4 was observed. Column, mean of independent triplicate experiments; error bars, SD.

Discussion

In this present study, we have shown that afatinib, a pan-HER inhibitor, was effective against an ESCC cell line with an activating EGFR mutation and an HNSCC cell line with an activating HER4 mutation. Of note, the function of the HER4 G1109C mutation was previously unclear. To the best of our knowledge, this is the first study to show that the HER4 G1109C mutation is an activating oncogenic mutation with a transformational ability and that afatinib is effective against an HER4 G1109C-mutated cell line.

The HER family, as well as their ligands, are often dysregulated directly sequenced. The KYSE50 cell line was used as a control. After 3 hours of exposure to afatinib, the phosphorylation levels of HER4, AKT, and ERK1/2 were reduced in a dose-dependent manner (0, 0.01, and 0.1 μmol/L; Fig. 5A). In contrast, these expressions were not changed after exposure to afatinib, the phosphorylation levels of HER4, AKT, and ERK1/2 was seen in the OSC-19 cell line (Fig. 5B). We also found that treatment with 0.1 μmol/L afatinib for 24 hours markedly increased the expressions of apoptosis-related molecules (cleaved caspase-3 and cleaved PARP) in the SAS cell line (Fig. 5A). In contrast, these expressions were not changed after treatment with afatinib in the OSC-19 cell line (Fig. 5B). These findings suggest that apoptosis is induced in response to afatinib in the SAS cell line carrying the activating HER4 G1109C mutation through the inhibition of the HER4 signal.

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To further investigate the in vivo efficacy of afatinib against tumor growth, a suspension of SAS cells was subcutaneously inoculated into the flank of each mouse. One week after tumor cell implantation, the tumors had achieved an average volume of approximately 100 to 150 mm³ and the treatment was initiated similarly to the KYSE270 cell line. The tumors from the SAS cell line were reduced by treatment with afatinib in a dose-dependent manner (vehicle: 1,433.3 ± 200.2 mm³ vs. afatinib 5 mg/kg: 931.0 ± 265.5 mm³, *P = 0.011) or vs. afatinib 15 mg/kg: 507.5 ± 173.8 mm³, *P < 0.0001). afatinib 5 mg/kg vs. afatinib 15 mg/kg, *P = 0.021; Fig. 5C).

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therapeutics. These receptors homo- and/or heterodimerize, leading to their activation by tyrosine kinase phosphorylation. Regulatory approval of small molecule (i.e., gefitinib, erlotinib, lapatinib, and afatinib) and antibody (i.e., trastuzumab, cetuximab, panitumumab, and pertuzumab) therapeutics directed against the HER family has been obtained for the treatment of breast, colorectal, NSCLC, and HNSCC. Afatinib is a highly selective, potent, and irreversible inhibitor of EGFR, HER2, and HER4 kinases (17, 18). Afatinib has preclinical antitumor activity in several models, especially as an EGFR inhibitor (17, 35), and patients with EGFR-mutated NSCLC dramatically respond to afatinib (19, 20). Similarly, in the present study, the KYSE270 cell line with an EGFR L861Q mutation was hypersensitive to afatinib. This is consistent with several previous studies showing that this mutation is an activating oncogenic mutation (16, 33, 34). This cell line, however, was not sensitive to erlotinib, a reversible EGFR inhibitor. Previous studies have shown that reversible EGFR-TKIs are less effective and irreversible EGFR-TKIs are more effective against cells carrying the EGFR L861Q mutation (33, 34, 37, 38), which is compatible with our data. Furthermore, our study has shown that the SAS cell line with the HER4 G1109C mutation is sensitive to afatinib, which also has an inhibitory effect on HER4, and that this mutation is an activating mutation with a transformational ability and tumorigenicity. HER4-knockdown significantly reduced the cellular growth in the SAS cell line, suggesting that this cell line partially depends on the HER4 signal for its cellular growth. These results provide a novel insight regarding afatinib as an HER4 inhibitor for HER4-mutated cancers. A recent phase III study of afatinib for HNSCC demonstrated a response rate of 10% (21), and a database has shown that 5% of...
investigate cellular growth, an MTT assay was performed using transfectant HEK293 cell lines with 1% FBS. The E317K (2.4) cell lines, compared with those in the controls (EGFP and HER4 wild-type). The relative expressions of phosphorylated HER4/HER4 were elevated in the HEK293/HER4 G1109C (1.8), HEK293/HER4 E317K (1.6), NIH3T3/HER4 G1109C (2.6), and NIH3T3/HER4 E317K (2.4) cell lines, compared with those in the controls (HER4 wild-type). B, β-actin was used as an internal control. C, cellular growth. To investigate cellular growth, an MTT assay was performed using transfectant HEK293 cell lines with 1% FBS. The HER4 E317K mutation was used as a positive control. The cellular growth of the HEK293/HER4 G1109C cell line was enhanced similar to that of the HEK293/HER4 E317K cell line, compared with controls. The enhanced cellular growth was cancelled by afatinib (0.01 μmol/L). Column, mean of independent triplicate experiments; error bars, SD; n.s., not significant; *, P < 0.05. C, transformational ability and tumorigenicity. To investigate the transformational abilities and tumorigenicities of the mutation, we used a focus formation assay and a tumorigenicity assay with the NIH3T3 cell line. Transfectant NIH3T3 cell lines were cultured for 2 to 3 weeks and photographed, and transfectant NIH3T3 cells (1 × 10^5) were injected subcutaneously into the right flank of nude mice; tumor formation was then examined 2 weeks after injection. The HER4 E317K mutation was used as a positive control, and the HER4 G1109C mutation had a transformational ability and a tumorigenicity (5/5) similar to that of the positive control (5/5). The NIH3T3/EGFP and NIH3T3/HER4 WT cell lines did not exhibit the formation of a focus or tumor (0/5 and 0/5, respectively); scale bar, 50 μm. D, HER4-knockdown in the cell lines. To investigate the role of the HER4 signal in the SAS cell line, the HER4 gene was subjected to knockdown using siRNA. We used two siRNAs for HER4 (HER4-1 and HER4-2) and a nonspecific target (scramble-1 and scramble-2) to avoid off-target effects. The cellular growth of the SAS cell line was significantly reduced by HER4-knockdown (less than 50% relative to the control), whereas that of the OSC-19 cell line was slightly reduced (more than 80% relative to the control). Column, mean of independent triplicate experiments; error bars, SD; *, P < 0.05.

Figure 4. Role of HER4 G1109C mutation in the SAS cell line. A, HER4 expression and phosphorylation in the transfectant cell lines. To address the role of the HER4 G1109C mutation, HER4-overexpressed cell lines were created (wild-type, G1109C, or E317K). The HER4 E317K mutation was used as a positive control. Each HER4 gene was equally introduced into each cell line, and HER4 was remarkably phosphorylated in the HEK293/HER4 G1109C, HEK293/HER4 E317K, NIH3T3/HER4 G1109C, and NIH3T3/HER4 E317K cell lines, compared with the controls (EGFP and HER4 wild-type). The relative expressions of phosphorylated HER4/HER4 were elevated in the HEK293/HER4 G1109C (1.8), HEK293/HER4 E317K (1.6), NIH3T3/HER4 G1109C (2.6), and NIH3T3/HER4 E317K (2.4) cell lines, compared with those in the controls (HER4 wild-type). B, β-actin was used as an internal control. C, cellular growth. To investigate cellular growth, an MTT assay was performed using transfectant HEK293 cell lines with 1% FBS. The HER4 E317K mutation was used as a positive control. The cellular growth of the HEK293/HER4 G1109C cell line was enhanced similar to that of the HEK293/HER4 E317K cell line, compared with controls. The enhanced cellular growth was cancelled by afatinib (0.01 μmol/L). Column, mean of independent triplicate experiments; error bars, SD; n.s., not significant; *, P < 0.05. C, transformational ability and tumorigenicity. To investigate the transformational abilities and tumorigenicities of the mutation, we used a focus formation assay and a tumorigenicity assay with the NIH3T3 cell line. Transfectant NIH3T3 cell lines were cultured for 2 to 3 weeks and photographed, and transfectant NIH3T3 cells (1 × 10^5) were injected subcutaneously into the right flank of nude mice; tumor formation was then examined 2 weeks after injection. The HER4 E317K mutation was used as a positive control, and the HER4 G1109C mutation had a transformational ability and a tumorigenicity (5/5) similar to that of the positive control (5/5). The NIH3T3/EGFP and NIH3T3/HER4 WT cell lines did not exhibit the formation of a focus or tumor (0/5 and 0/5, respectively); scale bar, 50 μm. D, HER4-knockdown in the cell lines. To investigate the role of the HER4 signal in the SAS cell line, the HER4 gene was subjected to knockdown using siRNA. We used two siRNAs for HER4 (HER4-1 and HER4-2) and a nonspecific target (scramble-1 and scramble-2) to avoid off-target effects. The cellular growth of the SAS cell line was significantly reduced by HER4-knockdown (less than 50% relative to the control), whereas that of the OSC-19 cell line was slightly reduced (more than 80% relative to the control). Column, mean of independent triplicate experiments; error bars, SD; *, P < 0.05.

HNSCC carry HER4 mutations (4). The biologic functions of these identified HER4 mutations remain unknown, and there does not seem to be any recurrence or hotspot (Supplementary Fig. S4). Some of these identified mutations are, however, located close to previously reported activating HER4 mutations in melanoma, which are located in various domains including a growth factor receptor, receptor L, and tyrosine kinase domains (36). Therefore, some of the patients who responded to afatinib might have such activating HER4 mutations.

The detailed molecular mechanism underlying the oncogenic activation and cellular transformation induced by the HER4 G1109C mutation (C-terminal) is unknown. Our experiments suggest that this mutation is not associated with the response to an HER4 ligand. Several studies have shown that other HER family gene mutations occurring within the C-terminal domain, such as EGFR C-terminal deletion mutations, have oncogenic activity and transformational ability and that they are effectively inhibited by EGFR-targeted drugs (39, 40). These findings suggest that constitutive asymmetric dimerization may be one of the possible mechanisms of oncogenic activation (39, 40). These studies suggest that the HER4 G1109C mutation might result in a mechanism similar to that of the C-terminal deletion mutations. In addition, the phosphorylation levels of other HER family members were enhanced in the HEK293/HER4 G1109C cell line suggesting that G1109C-mutated HER4 can activate other HER family members as a heterodimer.

The chimeric anti-EGFR mAb cetuximab was the first molecularly targeted therapy to receive approval for the treatment of HNSCC. The therapeutic effect of this mAb is exerted by its binding to the extracellular domain of EGFR, thereby
preventing ligands from activating EGFR while promoting EGFR internalization and antibody-dependent cell-mediated cytotoxicity (ADCC; 14). Afatinib activity has been seen in cetuximab refractory patients in clinical trials, suggesting a lack of cross-resistance in some instances (21, 41). Similarly, our present study has shown that afatinib is effective for cetuximab-resistant cell lines. Cetuximab, of course, cannot inhibit the HER4 signal, and previous studies using EGFR-mutated NSCLC cell lines have demonstrated that cetuximab does not inhibit their growth in vitro, probably because it does not inhibit the phosphorylation of EGFR (42). In addition, the EGFR mutation status was not a predictive biomarker for the efficacy of chemotherapy plus cetuximab in an NSCLC phase III trial (FLEX; ref. 43). These findings suggest that afatinib is effective against tumors that are resistant to cetuximab.

The possibility of EGFR amplification as a predictive biomarker for the efficacy of afatinib against HNSCC has been demonstrated in a previous study (35). In contrast, our present study has revealed that cell lines with EGFR or HER4 mutation (KYSE270 and SAS) are more sensitive to afatinib than those with EGFR amplification (KYSE30 and OSC-20). Whether EGFR mutation or amplification was a predictive biomarker for EGFR-TKIs against NSCLC had been debated, resulting in the EGFR mutation recently (44). Although predictive biomarkers for afatinib in ESCC or HNSCC remain unclear, these mutations are candidates, considering the results of our present study.

In conclusion, we have identified an ESCC and an HNSCC cell line that are hypersensitive to afatinib, one with a previously reported activating EGFR L861Q mutation and the other with an HER4 G1109C mutation with a previously unknown function.

Figure 5.
Western blot analyses in the HNSCC cell lines and the xenograft study. A, HER4 and its downstream signals. To examine the phosphorylation levels after afatinib exposure (0, 0.01, 0.1 μmol/L), we used the SAS (HER4 G1109C) and OSC-19 (HER4 wild-type) cell lines and the samples were collected 5 hours after stimulation. Afatinib induced a great decrease in the phosphorylation levels of HER4, AKT, and ERK1/2 in the SAS cell line, compared with the OSC-19 cell line. β-Actin was used as an internal control. B, apoptosis-related molecules. When apoptosis-related molecules were examined after afatinib exposure (0.1 μmol/L), the samples were collected 24 hours after stimulation in the SAS (HER4 G1109C) and OSC-19 (HER4 wild-type) cell lines. Afatinib greatly increased the expression of cleaved PARP and cleaved caspase-3 in the SAS cell line, compared with the OSC-19 cell line. β-Actin was used as an internal control. C, a xenograft study. We used the SAS cell line (HER4 G1109C) in the xenograft study. A suspension of 1 × 10⁶ cells (in 50 μL of PBS) with 50 μL of Matrigel was subcutaneously inoculated into the right flank of each nude mouse (n = 5). In the treatment groups, afatinib (5 or 15 mg/kg) was administered by oral gavage daily for 2 weeks; the control animals received 0.5% methylcellulose-0.4% polysorbate-80 as the vehicle. The tumors decreased in size after afatinib exposure in a dose-dependent manner [vehicle, 1,433.3 ± 200.2 mm³ vs. afatinib 5 mg/kg: 931.0 ± 265.5 mm³ (*, P = 0.011) or vs. afatinib 15 mg/kg: 507.5 ± 173.8 mm³ (*, P < 0.0001); afatinib 5 mg/kg vs. afatinib 15 mg/kg, P = 0.021*]. Lines, mean of 5 mice; error bars, SD; *, P < 0.05.
Table 1. HER family gene alterations in a database of esophageal or head-and-neck squamous cell carcinoma (ESCC or HNSCC)

<table>
<thead>
<tr>
<th>Cancer type</th>
<th>Dataset</th>
<th>Gene</th>
<th>Mutation</th>
<th>Amp</th>
<th>Del</th>
<th>Multiple</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESCC</td>
<td>ICGC</td>
<td>EGF R</td>
<td>1/88 (1.3%)</td>
<td>34/140 (24.3%)</td>
<td>NA</td>
<td>NA</td>
<td>(1)</td>
</tr>
<tr>
<td></td>
<td>HER2</td>
<td>0/88 (0.0%)</td>
<td>12/140 (8.6%)</td>
<td>NA</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HER3</td>
<td>1/88 (1.3%)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HER4</td>
<td>0/88 (0.0%)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HNSCC Broad</td>
<td>EGF R</td>
<td>2/74 (2.7%)</td>
<td>5/69 (7.2%)</td>
<td>0/69 (0%)</td>
<td>NA</td>
<td>(31)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HER2</td>
<td>2/74 (2.7%)</td>
<td>0/69 (0%)</td>
<td>NA</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HER3</td>
<td>1/74 (1.4%)</td>
<td>0/69 (0%)</td>
<td>NA</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HER4</td>
<td>1/74 (1.4%)</td>
<td>1/69 (1.4%)</td>
<td>0/69 (0%)</td>
<td>NA</td>
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<tr>
<td>HNSCC JHU</td>
<td>EGF R</td>
<td>0/32 (0.0%)</td>
<td>4/42 (9.5%)</td>
<td>NA</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HER2</td>
<td>1/32 (3.3%)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HER3</td>
<td>0/32 (0.0%)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HER4</td>
<td>1/32 (3.3%)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HNSCC TCGA</td>
<td>EGF R</td>
<td>9/279 (3.2%)</td>
<td>26/279 (9.3%)</td>
<td>1/279 (0.4%)</td>
<td>4/279 (1.4%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HER2</td>
<td>5/279 (1.8%)</td>
<td>6/279 (2.2%)</td>
<td>0/279 (0.0%)</td>
<td>0/279 (0.0%)</td>
<td>(4)</td>
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</tr>
<tr>
<td></td>
<td>HER3</td>
<td>8/279 (2.9%)</td>
<td>2/279 (0.7%)</td>
<td>0/279 (0.0%)</td>
<td>0/279 (0.0%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HER4</td>
<td>14/279 (5.0%)</td>
<td>0/279 (0.0%)</td>
<td>3/279 (1.1%)</td>
<td>0/279 (0.0%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: Amp, amplification; Del, deletion; ICGC, International Cancer Genome Consortium; JHU, Johns Hopkins University; TCGA, The Cancer Genome Atlas; NA, data were not available.

*Amplification data were analyzed from Supplementary Table S21 of the original paper. No data were available for HER3 or HER4 amplification or HER family gene deletion.

**Gene copy numbers were analyzed from Supplementary Table S11 of the original article.**

**EGFR amplification data were analyzed from Supplementary Table S7 of the original article. The other amplification and deletion data were not available.**

neither of which result in the amplification of HER family genes. Our experimental findings revealed that theHER4 G1109C mutation is an activating oncogenic mutation with a transformational ability and that afatinib was effective against an HER4 G1109C-mutated HNSCC cell line by inhibiting the HER4 signal, both in vitro and in vivo. Considering these findings, these mutations can be considered as predictive biomarker candidates for the efficacy of afatinib. To validate our findings, further research, including clinical sample analyses, is needed.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: Y. Nakamura, Y. Togashi, E. Banno, M. Terashima, S. Hamada, K. Nishio

Development of methodology: Y. Nakamura, Y. Togashi, H. Nakahara

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Nakamura, Y. Togashi, K. Sakai

Analysis and interpretation of data (e.g., statistical analysis, bioinformatics, computational analysis): Y. Nakamura, Y. Togashi, S. Tomida, H. Hayashi, M.A. de Velasco

Writing, review, and/or revision of the manuscript: Y. Nakamura, Y. Togashi, H. Nakahara, E. Banno, M. Terashima, H. Hayashi, M.A. de Velasco, T. Okegawa, K. Nutahara, S. Hamada, K. Nishio

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Togashi, E. Banno, M. Terashima, Y. Fujita

Study supervision: T. Okegawa, K. Nutahara, S. Hamada, K. Nishio

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References


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