Anti-tumor effect of neratinib against lung cancer cells harboring HER2 oncogene alterations

YUSUKE OGOSHI, KAZUHIKO SHIEN, TAKAHIRO YOSHIOKA, HIDEJIRO TORIGOE, HIROKI SATO, MASAKIYO SAKAGUCHI, SHUTA TOMIDA, KEI NAMBA, EISUKE KURIHARA, YUTA TAKAHASHI, KEN SUZAWA, HIROMASA YAMAMOTO, JUNICHI SOH and SHINICHI TOYOKA

Departments of 1Thoracic, Breast and Endocrinological Surgery, 2Cell Biology and 3Biobank, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama 700-8558, Japan

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Abstract. Human epidermal growth factor receptor 2 (HER2) is a member of the ErbB family of receptor tyrosine kinases. Numerous studies have reported the amplification and overexpression of HER2 in several types of cancer, including non-small cell lung cancer (NSCLC). However, the benefits of HER2-targeted therapy have not been fully established. In the present study, the anti-tumor effect of neratinib, an irreversible pan-HER tyrosine kinase inhibitor (TKI), against NSCLC cells harboring HER2 alterations was investigated. The sensitivity of normal bronchial epithelial cells (BEAS-2B) ectopically overexpressing wild-type or mutant HER2 to neratinib was assessed. Furthermore, the anti-tumor activity of neratinib in several NSCLC cell lines harboring HER2 alterations was determined in vitro and in vivo, and the association between their genetic alterations and sensitivity to neratinib treatment was investigated. BEAS-2B cells ectopically overexpressing wild-type HER2 or mutants (A775insYVMA, G776VC, G776LC, P780insGSP, V659E, G660D and S310F) exhibited constitutive autophosphorylation of HER2, as determined by western blotting. While these BEAS-2B cells were sensitive to neratinib, they were insensitive to erlotinib, a first-generation epidermal growth factor receptor-TKI. Neratinib also exerted anti-proliferative effects on HER2-altered (H2170, Calu-3 and H1781) NSCLC cell lines. Neratinib was also demonstrated to exert strong tumor growth inhibitory activity in mouse xenograft models using HER2-altered lung cancer cells. The results of the present study strongly suggest that neratinib has potential as a promising therapeutic option for the treatment of HER2-altered NSCLC.

Introduction

Recent progress in the identification of tumor-specific molecular alterations has contributed to novel therapeutic approaches, and the development of molecular-targeting anti-tumor drugs has improved patient survival. For example, epidermal growth factor receptor (EGFR)-targeted therapy for non-small cell lung cancer (NSCLC) cases harboring EGFR oncogenic alterations is a promising strategy for improving the clinical outcome of patients with NSCLC (1,2).

Human epidermal growth factor receptor 2 (HER2) is part of the ErbB family of receptor tyrosine kinases. HER2 is activated by homodimerization or heterodimerization with other receptors in the ErbB family, particularly EGFR (3). HER2 has important roles in pathogenesis of certain types of human cancer, and numerous studies have reported the amplification and overexpression of HER2 in cancer, particularly breast cancer (4-6). The reported frequencies of HER2 overexpression and HER2 amplification in NSCLC range from 11-32 and 2-23%, respectively (7-10). HER2 mutations have been identified in 2-4% of all NSCLCs, and are usually mutually exclusive with other driver mutations (11,12). Several HER2 variants have been reported previously, the majority of which are in-frame insertions in exon 20 of the kinase domain, including A775insYVMA, G776VC, P780insGSP, V659E, G660D and G776LC (12). Our previous study identified two novel mutations in the HER2 transmembrane domain, which is encoded by exon 17 (V659E and G660D), as rare HER2 variants in lung adenocarcinoma, and the preliminary data suggested that these mutations may be oncogenic (13). An extracellular domain point mutation, S310F, in exon 8 has also been reported to be oncogenic (14). However, the benefit of HER2-targeted therapy against NSCLC harboring HER2 alterations is far less well defined than the known benefit against breast cancer and gastric cancer with HER2 alterations (15).

Afatinib (BIBW 2992) is a pan-HER tyrosine kinase inhibitor (TKI) that has been approved for the treatment of patients with NSCLC harboring EGFR mutations. Recently,
afatinib has attracted attention as a HER2-targeting treatment agent. Afatinib was reported to exhibit good clinical activity in patients with lung adenocarcinoma carrying HER2 mutations (16,17). In preclinical studies, afatinib inhibited the growth of HER2-altered NSCLC cells (18).

Neratinib (HKI-272) is another pan-HER TKI that has been reported to improve the overall survival of post-operative patients with HER2-positive breast cancer previously treated with trastuzumab-based adjuvant therapy (19). A phase II clinical trial (PUMA-HER-4201) evaluating the usefulness of neratinib combined with the mechanistic target of rapamycin kinase inhibitor temsirolimus for patients with NSCLC harboring HER2-mutations (insertions in exon 20) in currently ongoing; patient accrual has been completed, and the final results of the trial are being awaited (20,21). Thus far, the available data is limited, and the benefits of neratinib treatment remain unclear, particularly for cases with relatively rare mutations. Therefore, the aim of the present study was to investigate the potential use of neratinib against HER2-altered NSCLC, including cases with relatively uncommon mutations.

Materials and methods

Cell lines and reagents. Four lung cancer cell lines (A549, Calu-3, NCI-H2170 and NCI-H1781) and one normal human bronchial epithelial cell line (BEAS-2B) were used in the current study. Calu-3, H2170 and H1781 cells were received as gifts from Dr Adi F. Gazdar (University of Texas Southwestern Medical Center at Dallas, Dallas, TX, USA) (22,23). A549 was purchased from American Type Culture Collection (Manassas, VA, USA). BEAS-2B was purchased from European Collection of Authenticated Cell Cultures (Public Health England, Porton Down, UK). All the cancer cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), and the BEAS-2B cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS. They were cultured in a humidified atmosphere containing 5% CO2 at 37°C. Neratinib and erlotinib were purchased from Selleck Chemicals (Houston, TX, USA).

Plasmid constructs and transfection. Human cDNAs encoding full-length HER2 (wild-type and its variants, A775insYVMA, G776VC, G776LC, P780insGSP, V659E, G660D and S310F) were inserted into the pIDT-SMART (C-TSC) vector, pCMVIRTSC (24). Transient transfection of the BEAS-2B cells with the mammalian expression vectors was performed using Lipofectamine® 3000 (Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer’s protocol.

Western blot analysis and immunohistochemistry. Total cell lysates were extracted using a mixture of radioimmunoprecipitation assay lysis buffer, phosphatase inhibitor cocktails 2 and 3 (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and Complete Mini protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland). Western blot analysis was performed using the conventional method with the following primary antibodies: Anti-EGFR (4267S; 1:1,000), phospho-(p-) EGFR (Tyr1068) (3777S; 1:1,000), HER2 (4290S; 1:1,000), p-HER2 (Tyr1221/1222) (2243S; 1:1,000), Akt (9272S; 1:1,000), p-Akt (Ser473) (4060S; 1:1,000), p44/p42 mitogen-activated protein kinase (MAPK) (9102S; 1:1,000), p-p44/p42 MAPK (4370S; 1:1,000), cleaved poly (ADP-ribose) polymerase (PARP; Asp214) (5625S; 1:1,000) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). β-actin (used as loading control) (MAB1501R; 1:1,000) was purchased from EMD Millipore (Billerica, MA, USA). The secondary antibodies were horseradish peroxidase-conjugated anti-mouse IgG (sc-2031; 1:2,500) or anti-rabbit IgG (sc-2030; 1:2,500; Santa Cruz Biotechnology, Inc., Dallas, TX, USA).

To detect specific signals, the membranes were examined using the ECL Prime Western Blotting Detection System (GE Healthcare Life Sciences, Little Chalfont, UK) and a LAS-3000 imager (Fujifilm, Tokyo, Japan). Immunohistochemical staining with anti-p-HER2 (1:300 Y1221/1222; Cell Signaling Technology, Inc.) was conducted. The detailed protocol for the immunohistochemical staining has been described previously (25).

Cell growth inhibition assay. Cells were cultured with or without the appropriate drugs for 72 h and the sensitivities of the cells to the drugs were determined via a modified MTS assay using Cell Titer 96 Aqueous One Solution Reagent (Promega Corporation, Madison, WI, USA), as described previously (26). The anti-proliferative activity of each drug is presented as the IC50, which is the concentration of the drug required to inhibit cell proliferation by 50%.

Cell cycle analysis. The effects of neratinib on the cell cycle distribution were assessed using a propidium iodide staining-based assay (CycleTEST PLUS DNA reagent kit; BD Biosciences, Franklin Lakes, NJ, USA) and a BD Accuri C6 flow cytometer (BD Biosciences). Doubles, cell debris and fixation artifacts were gated out, and cell cycle analysis was performed.

Xenograft model. NOD/SCID female mice [n=18, body weight (mean ± standard)=19.9±0.6 g, 6-weeks-old] were purchased from Charles River Laboratories, Inc. (Wilmington, MA, USA). All the mice were provided with sterilized food and water, and housed in a barrier facility under a 12:12 h light/dark cycle. Each cell line (5x106 cells) was suspended in 200 µl RPMI-1640 medium mixed with Matrigel Basement Membrane Matrix (Corning Incorporated, Corning, NY, USA) and subcutaneously injected into the backs of the mice. The tumor volume was calculated using the empirical formula, \( V = \frac{1}{2} \times \text{[shortest diameter]}^2 \times \text{[longest diameter]} \). When the tumor volume exceeded ~50 mm³, the mice were orally administered with vehicle alone or neratinib (40 mg/kg, 6 days a week). Neratinib was prepared in 0.5 w/v% (methyl cellulose. The tumor volume was measured three times a week using callipers. After 4 weeks of treatment, or when humane endpoints were reached, mice were euthanized by cervical dislocation.

All animal experiments were performed in accordance with protocols approved by the Animal Care and Use Committee of Okayama University (Okayama, Japan; permission no. OKU-2018215) and were conducted in accordance with recent legislation of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.
Statistical analysis. Statistical analysis was performed using EZR software (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria). EZR is a modified version of R commander software (version 1.6-3) with additional statistical functions frequently used in biostatistics (27). Data from two groups were compared using t-test. All tests were two-sided. P<0.05 was considered to indicate a statistically significant difference.

Results

HER2 mutations activate HER2 signaling, which is inhibited by neratinib. To examine the effect of HER2 alterations on the signal transduction pathways, normal bronchial epithelial cells (BEAS-2B) were transiently transfected with vectors containing wild-type HER2 or one of seven HER2 mutations: Four kinase domain mutations (A775insYVMA, G776VC, G776LC, and P780insGSP), two transmembrane domain mutations (V659E and G660D) and one extracellular domain mutation (S310F).

The sensitivity of BEAS-2B cells ectopically expressing wild-type or mutant HER2 to erlotinib (an EGFR-TKI) or neratinib (a pan-HER-TKI) was examined. At 48 h after transfection, the cells were cultured in the presence or absence of erlotinib or neratinib for 6 h. Erlotinib had minimal effect on the phosphorylation of HER2 and EGFR, whereas neratinib strongly inhibited the phosphorylation of HER2 and EGFR compared with untreated cells (Fig. 1; Table I). The cell lines in this panel consisted of two HER2-amplified NSCLC cell lines (H2170 and Calu-3) and one HER2-mutant NSCLC cell line (H1781). The detailed HER2 genetic profiles of these three cell lines are presented in Table I based on the results of a previous study (18). The proliferation of the two HER2-amplified lung cancer cell lines, H2170 and Calu-3, was inhibited by neratinib, with IC$_{50}$ values of 4.7 and 16.5 nM, respectively. Neratinib also exerted a strong cytotoxic effect against the H1781 cells, with an IC$_{50}$ of 13.6 nM. By contrast, the H2170 and H1781 cell lines were resistant to erlotinib treatment, with IC$_{50}$ values of 1150 and 1080 nM, respectively. Calu-3 cells were partially sensitive to erlotinib, with an IC$_{50}$ of 316 nM. These results were consistent with those of a previous report (Table I; Fig. 2A) (28).

Subsequently, the effect of neratinib on signal transduction pathways in HER2-amplified or HER2-mutant lung cancer cells was assessed. After 6 h of treatment with neratinib, the cells were lysed and then subjected to western blot analysis. As demonstrated in Fig. 2B, neratinib potentely inhibited the phosphorylation of HER2 and EGFR when administered at concentrations as low as 0.01 µM, and downstream signals, including phosphorylation of Akt and MAPK, were also inhibited by neratinib in the HER2-amplified and the HER2-mutant lung cancer cells. Taken together, these results suggest that neratinib has strong anti-tumor activity against HER2-amplified and HER2-mutant lung cancer cells in vitro.

Neratinib induces cell cycle arrest and apoptosis in HER2-dependent cells. The effect of neratinib on the cell cycle and apoptosis in HER2-driven cells (H2170, Calu-3 and H1781) and KRAS mutant cells (A549) was also examined to determine the mechanism of growth inhibition. Cells were treated with 0.1 µM neratinib for 48 h and then analyzed using flow cytometry. To assess the cell cycle distribution, the sub-G1 fraction was excluded and the percentage of cells in each cycle phase was measured (Fig. 3A). Neratinib treatment caused an increase in the number of H2170, Calu-3 and H1781 cells in G1 phase compared with the distribution

|               | WT | A775 InsYVMA | G776VC | G776LC | P780 InsGSP | V659E | G660D | S310F |
|---------------|----|--------------|--------|--------|-------------|-------|-------|-------|
| **Erlotinib (1 µM)** |   | -            | -      | -      | +           | +     | +     | +     |
| **Neratinib (0.1 µM)** |   | +            | +      | +      | +           | +     | +     | +     |

Neratinib inhibits the growth of HER2-amplified and HER2-mutant lung cancer cells. The anti-tumor activity of neratinib (a pan-HER-TKIs) and erlotinib (an EGFR-TKI) against HER2-driven NSCLC cell lines was subsequently examined (Table I; Fig. 2B).
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Table I. Characteristics and IC\textsubscript{50} values for pan-HER tyrosine kinase inhibitor and EGFR tyrosine kinase inhibitor in non-small cell lung cancer cell lines.

| Cell line | Histologic subtype | Gene copy number | Mutation status | Genetic alteration | IC\textsubscript{50} (nM) |
|-----------|--------------------|------------------|-----------------|-------------------|-----------------|
| H2170     | SQ                 | 2                | WT              | HER2 amplification | 4.7             |
|           |                    | 95               | WT              |                   | 1,150           |
| Calu-3    | AD                 | 4                | WT              | HER2 amplification | 16.5            |
|           |                    | 111              | WT              |                   | 316             |
| H1781     | AD                 | 2                | WT              | G776VC            | 13.6            |
|           |                    | 3                | WT              | HER2 mutation     | 1,080           |

SQ, squamous cell carcinoma; AD, adenocarcinoma; IC\textsubscript{50}, half-maximal inhibitory concentration; WT, wild-type; HER2, human epidermal growth factor receptor 2; EGFR, epidermal growth factor receptor.

in untreated cells; a similar increase was not detected in A549 cells. Subsequently, western blotting was performed to evaluated cell apoptosis, using cleaved PARP antibody as an apoptosis marker. Neratinib induced apoptosis in the H2170, Calu-3 and H1781 cells; however, an increase in cleaved PARP was not detected in A549 cells following neratinib treatment (Fig. 3B). These results suggest that neratinib induces anti-proliferative effects via G1 arrest and apoptotic cell death in HER2-altered cells (H2170, Calu-3 and H1781 cells), whereas HER2-independent NSCLC cells were not sensitive to neratinib.

Anti-tumor effect of neratinib in a mouse xenograft model of HER2-altered lung cancer. Based on the in vitro data, the anti-tumor effect of neratinib was investigated using mouse xenograft models of HER2-driven lung cancer. Two HER2-amplified (H2170 and Calu-3) cell lines and one HER2-mutant (H1781) cell line were used for this experiment. The dose of neratinib was selected based on the results of previous reports (29,30). Once the xenograft tumor volume reached ~50 mm\(^3\), the mice were orally treated with the vehicle alone or neratinib (40 mg/kg, 6 days a week). As demonstrated in Fig. 4, neratinib treatment significantly inhibited the tumor
growth of the H2170, Calu-3 and H1781 xenografts compared with the vehicle control (P<0.001). In the H2170 xenograft group treated with the vehicle control, the mice were sacrificed on day 18, as the tumor volumes had reached the humane endpoint (~2,000 mm$^3$). The levels of p-HER2 in the xenografts were examined using immunohistochemistry. The level of p-HER2 was suppressed in tumors from mice treated with neratinib compared those that received the vehicle control (Fig. 4G-I).

Discussion

The findings of the current study demonstrated the in vivo and in vitro anti-tumor efficacy of neratinib against lung cancer cells harboring HER2 alterations. Neratinib is an irreversible human EGFR-TKI that also binds to the tyrosine kinase domains of HER2 and HER4. In patients with breast cancer, neratinib treatment has improved overall survival among post-operative patients with HER2-positive breast cancer previously treated with trastuzumab, and neratinib has been approved for extended adjuvant treatment in patients with early-stage HER2-positive breast cancer (19). By contrast, only a few reports have discussed the efficacy of neratinib against NSCLC. Furthermore, recent advances in clinical tumor sequencing have identified a large number of variants in oncogenic driver genes with unknown significance, including EGFR, anaplastic lymphoma kinase and HER2 variants. Heterogeneity among the functions and/or drug sensitivities of tumors with these mutations has been reported. Notably, suitable TKI selection for patients with individual oncogene variants, including relatively rare mutations, has been proposed (31). Thus, further preclinical and clinical investigations of patients with tumors harboring uncommon HER2 gene variants are warranted.

In the current study, neratinib inhibited the growth of cells harboring HER2 mutations in the transmembrane domain, extracellular domain and kinase domain. Although neratinib has been previously reported to inhibit the growth of cells harboring HER2 mutations in the kinase domain, to the best of our knowledge, there are no reports of neratinib inhibiting the growth of cells with HER2 mutations in the transmembrane and extracellular domains (32). Genomic and functional analyses have suggested that mutations in the transmembrane domain of HER2 encoded by exon 17 (V659E and G660D) are oncogenic in lung adenocarcinoma. Additionally, although the variant rate is low, mutations in the extracellular domain (such as S310F) are also considered to be oncogenic alterations in lung adenocarcinoma (14). For cells harboring mutations in the extracellular domain, anti-HER2 monoclonal antibodies, such as trastuzumab, can target this region of the receptor and prevent homo-dimerization and receptor activation (33). However, because the kinase domain is constitutively activated in tumors harboring kinase domain mutations, the anti-proliferative effects of monoclonal antibodies may be limited even if dimerization is inhibited (34). The effect of trastuzumab and other antibodies may also be
limited in tumors harboring mutations in the transmembrane domain, as HER2 dimerization is thought to be stable even if trastuzumab or other antibodies bind to the extracellular domain (35). On the other hand, neratinib targets the kinase domain of HER2 and inhibits the phosphorylation and activity of HER receptors, and therefore may have a therapeutic advantage over trastuzumab and other monoclonal antibodies, as neratinib can exert anti-tumor effects regardless of the domain in which the mutations exist, as demonstrated in the present report. Although HER2 mutations in the extracellular domain are rare (36), neratinib may be a useful therapeutic option in patients with such mutations.

The findings of the present study demonstrated the efficacy of neratinib against NSCLC cell lines harboring several HER2 variants. The anti-tumor activity of neratinib was also evaluated in a mouse xenograft model using lung cancer cells with HER2 amplification or HER2 mutation. To the best of our knowledge, this is the first report of in vivo experiments using neratinib for mouse xenograft models of lung cancer cells harboring HER2 amplification or HER2 mutations.

In conclusion, the anti-tumor effect of neratinib against lung cancers harboring HER2 alterations was demonstrated in vitro and in vivo. The findings suggest that neratinib has potential as a promising therapeutic option for the treatment of HER2-altered NSCLC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YO, KSh, KSu, JS, HY and SToy conceived and designed the study. YO, KSh, and MS performed majority of the in vitro experiments. MS performed cell transfection. YO, YT, STom...
and KSh performed the statistical analysis. JS, HY, TY, HT, HS, EK and KN participated in the animal experiments and helped perform the analysis with constructive discussion. MS, STom, KSu, KSh and SToy supervised the study.

Ethics approval and consent to participate

All animal experiments were performed in accordance with protocols approved by the Animal Care and Use Committee of Okayama University (Okayama, Japan; permission no. OKU-2018215) and were conducted in accordance with recent legislation of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

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References

1. Mok TS, Wu YL, Thongprasert S, Yang CH, Chu DT, Saigo N, Sumpawarat P, Han B, Margono B, Ichinose Y, et al: Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma. N Engl J Med 361: 947-957, 2009.

2. Reck M, Heigener DF, Mok T, Soria JC and Rabe KF: Management of non-small-cell lung cancer: Recent developments. Lancet 362: 709-717, 2003.

3. Oxnard GR, Binder A and Janne PA: New targetable oncogenes in non-small-cell lung cancer. J Clin Oncol 31: 1097-1104, 2013.

4. Youlend DR, Crabb SM and Baade PD: The international epidemiology of lung cancer: Geographical distribution and secular trends. J Thorac Oncol 3: 819-831, 2008.

5. Moasser MM: The oncogene HER2: Its signaling and transforming functions and its role in human cancer pathogenesis. Oncogene 26: 6469-6487, 2007.

6. Yu D and Hung MC: Overexpression of ErbB2 in cancer and ErbB2-targeting strategies. Oncogene 19: 6115-6121, 2000.

7. Tan D, Deeb G, Wang J, Shocum HK, Winston J, Wiseman S, Beck A, Sait S, Anderson T and Nwogu C: HER-2/neu protein and receptor -targeted therapy in human breast cancer. Cancer 77: 162-167, 2012.

8. Dec Heer, Frank V, Di Marco P, Etsinger D and Weinberg RA: Real-time PCR analysis of HER2 mRNA expression in breast cancer by fluorescence in situ hybridization. Diagn Mol Pathol 12: 201-211, 2003.

9. Beck A, Sait S, Anderson T and Nwogu C: HER -2/neu protein and ErbB2-targeting strategies. Oncogene 19: 6115-6121, 2000.

10. Yu D and Hung MC: Overexpression of ErbB2 in cancer and ErbB2-targeting strategies. Oncogene 19: 6115-6121, 2000.

11. Yamamoto H, Higasa K, Sakaguchi M, Shien K, Soh J, Ichimura K, Furukawa M, Hashida S, Tsukuda K, Takigawa N, et al: Novel germline mutation in the transmembrane domain of HER2 in familial lung adenocarcinomas. J Natl Cancer Inst 106: djt338, 2014.

12. Greulich H, Kaplan B, Mertins P, Chen TH, Tanaka KE, Yun CH, Zhang X, Lee SH, Cho J, Ambrogio L, et al: Functional analysis of receptor tyrosine kinase mutations in lung cancer identifies oncogenic extracellular domain mutations of ERBB2. Proc Natl Acad Sci USA 109: 14476-14481, 2012.

13. Mar N, Vredenburgh JJ and Wasser JS: Targeting HER2 in the treatment of non-small-cell lung cancer. Lung Cancer 87: 220-225, 2015.

14. De Grève J, Teugels E, Geers C, Decoster L, Glaudemans D, Depuy J, Everaert H, Umelo I, Int Veld P and Schaller D: Clinical activity of afatinib (BIBW 2992) in patients with lung adenocarcinoma with mutations in the kinase domain of HER2/new. Lung Cancer 76: 123-127, 2012.

15. Mazieres J, Peters S, Lepage B, Cortot AB, Barlesi F, Beau-Faller M, Besse B, Blons H, Mansuet-Lupo A, Urban T, et al: Lung cancer that harbors an HER2 mutation: Epidemiologic characteristics and therapeutic perspectives. J Clin Oncol 31: 1997-2003, 2013.

16. Suzawa K, Toyooka S, Sakaguchi M, Morita M, Yamamoto H, Tomida S, Ohutsuka T, Watanabe M, Hashida S, Maki Y, et al: Antibody effect of afatinib, as a human epidermal growth factor receptor 2-targeted therapy, in lung cancers harboring HER2 oncogene alterations. Cancer Sci 107: 45-52, 2016.

17. Chan A, Delalosge S, Holmes FA, MoY, Ibawa H, Harvey VJ, Robert NJ, Silovski T, Gokmen E, Von Minckwitz G, et al: Neratinib after trastuzumab-based adjuvant therapy in patients with HER2-positive breast cancer (Exente): A multicentre, randomised, double-blind, placebo-controlled, phase 3 trial. Lancet Oncol 17: 367-376, 2016.

18. Gandhi L, Besse B, Mazieres J, Waqar S, Cortot A, Barlesi F, Quioz E, Otterson G, Ettinger D, Horn L, et al: MA04-02 Neratinib ± temsirolimus in HER2-mutant lung cancers: An international, randomized phase II study. J Thorac Oncol 12 (Suppl): S358-S359, 2017.

19. Mazieres J, Barlesi F, Filleron T, Besse B, Monnet I, Beau-Faller M, Peters S, Dansin E, Früh M, Pless M, et al: Lung cancer patients with HER2 mutations treated with chemotherapy and HER2-targeted drugs: results from the European EUHER2 cohort. Ann Oncol 27: 281-286, 2016.

20. Gandhi J, Zhang J, Xie Y, Soh J, Shigematsu H, Zhang W, Yamamoto H, Peyton M, Girard L, Lockwood WW, et al: Alterations in genes of the EGFR signaling pathway and their relationship to EGFR tyrosine kinase inhibitor sensitivity in lung cancer cell lines. PLoS One 4: e4576, 2009.

21. Girard L, Zöchbauer-Müller S, Virmani AK, Gazdar AF and Minna JD: Genome-wide allelotyping of lung cancer identifies new regions of allelic losses. Differences between small cell lung cancer and non-small cell lung cancer, and loci clustering. Cancer Res 60: 4894-4906, 2000.

22. Sakaguchi M, Watanabe M, Kinoshita R, Kaku H, Ueki H, Futami J, Murata H, Inoue Y, Li SA, Huang P, et al: Dramatic increase in expression of a transgene by insertion of promoters downstream of the cargo gene. Mol Biotechnol 56: 621-630, 2014.

23. Shien K, Toyooka S, Ichimura K, Soh J, Furukawa M, Maki Y, Muraoka T, Tanaka N, Ueno T, Asano H, et al: Prognostic impact of cancer stem cell-related markers in non-small cell lung cancer patients treated with induction chemoradiotherapy. Lung Cancer 77: 162-167, 2012.

24. Shien K, Toyooka S, Yamamoto H, Soh J, Jida M, Thu KL, Hashida S, Maki Y, Ichihara E, Asano H, et al: Acquired resistance to EGFR inhibitors is associated with a manifestation of stem cell-like properties in cancer cells. Cancer Res 73: 3051-3061, 2013.

25. Kanda Y: Investigation of the freely available easy-to-use software ‘EZR’ for medical statistics. Bone Marrow Transplant 48: 452-458, 2013.

26. Engelman JA, Janne PA, Meruelo C, Pearlberg J, Mukohara T, Plante C, Cichowski K, Johnson BE and Cantley LC: ErbB2 mediates phosphoinositol 3-kinase activity in gefitinib-sensitive non-small cell lung cancer cell lines. Proc Natl Acad Sci USA 102: 3788-3793, 2005.

27. Schwab CL, English DP, Black J, Bellone S, Lopez S, Cocco E, Watanabe R, Elzbieta B, Butkiewicz F, et al: SNMP shows efficacy in the treatment of HER2 amplified carcinosarcoma in vitro and in vivo. Gynecol Oncol 139: 112-117, 2015.
30. Menderes G, Bonazzoli E, Bellone S, Black JD, Lopez S, Pettinella F, Masserdotti A, Zammataro L, Litkouhi B, Ratner E, et al: Efficacy of neratinib in the treatment of HER2/neu-amplified epithelial ovarian carcinoma in vitro and in vivo. Med Oncol 34: 91, 2017.

31. Kohsaka S, Nagano M, Ueno T, Suehara Y, Hayashi T, Shimada N, Takahashi K, Suzuki K, Takamochi K, Takahashi F and Mano H: A method of high-throughput functional evaluation of EGFR gene variants of unknown significance in cancer. Sci Transl Med 9: pii: eaan6566, 2017.

32. Minami Y, Shimamura T, Shah K, LaFramboise T, Glatt KA, Liniker E, Borgman CL, Haringsma HJ, Feng W, Weir BA, et al: The major lung cancer-derived mutants of ERBB2 are oncogenic and are associated with sensitivity to the irreversible EGFR/ERBB2 inhibitor HKI-272. Oncogene 26: 5023-5027, 2007.

33. Tai W, Mahato R and Cheng K: The role of HER2 in cancer therapy and targeted drug delivery. J Control Release 146: 264-275, 2010.

34. Wang SE, Narasanna A, Perez-Torres M, Xiang B, Wu FY, Yang S, Carpenter G, Gazdar AF, Muthuswamy SK and Arteaga CL: HER2 kinase domain mutation results in constitutive phosphorylation and activation of HER2 and EGFR and resistance to EGFR tyrosine kinase inhibitors. Cancer Cell 10: 25-38, 2006.

35. Ou SI, Schrock AB, Bocharov EV, Klempner SJ, Haddad CK, Steinecker G, Johnson M, Giltiz BJ, Chung J, Campregher PV, et al: HER2 transmembrane domain (TMD) mutations (V659/G660) that stabilize homo- and heterodimerization are rare oncogenic drivers in lung adenocarcinoma that respond to Afatinib. J Thorac Oncol 12: 446-457, 2017.

36. Eng J, Hsu M, Chaft JE, Kris MG, Arcila ME and Li BT: Outcomes of chemotherapies and HER2 directed therapies in advanced HER2-mutant lung cancers. Lung Cancer 99: 53-56, 2016.

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