Inhibition of β-Catenin Enhances the Anticancer Effect of Irreversible EGFR-TKI in EGFR-Mutated Non–small-cell Lung Cancer with a T790M Mutation

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Introduction: Patients with non–small-cell lung cancer (NSCLC) with somatic activating mutations of the epidermal growth factor receptor gene (EGFR mutations) generally respond to EGFR tyrosine kinase inhibitors (EGFR-TKIs). β-Catenin is a key component of the Wnt/β-Catenin signal and is an important oncogene that is involved in the pathogenesis and progression of malignant tumors, especially cancer stem cells.

Methods and Results: We found that EGFR-mutated NSCLC cell lines exhibited a high expression level of β-Catenin, compared with cell lines with the wild-type EGFR gene, and XAV939 (a β-Catenin inhibitor) enhanced the sensitivities to EGFR-TKI in EGFR-mutated NSCLC cell lines. In EGFR-mutated NSCLC cell lines with the acquired resistance threonine-to-methionine mutation in codon 790 (T790M) mutation, XAV939 enhanced the sensitivity of the cells to an irreversible EGFR-TKI but not a reversible EGFR-TKI. The combination of XAV939 and EGFR-TKIs strongly inhibited the β-Catenin signal and strongly decreased the phosphorylation of EGFR, compared with the use of EGFR-TKIs alone, suggesting an interaction between EGFR and the β-Catenin signal. The stem cell-like properties of the EGFR-mutated cell line carrying the T790M mutation were inhibited by XAV939 and BIBW2992 (an irreversible EGFR-TKI). Furthermore, the stem cell-like properties were strongly inhibited by a combination of both the agents. A xenograft study demonstrated that β-Catenin knockdown enhanced the antitumor effect of BIBW2992 in the EGFR-mutated NSCLC cell line carrying the T790M mutation.

Conclusion: Our findings indicate that β-Catenin might be a novel therapeutic target in EGFR-mutated NSCLC carrying the T790M mutation.

Key Words: Non–small-cell lung cancer, Epidermal growth factor receptor T790M mutation, Epidermal growth factor receptor tyrosine kinase inhibitor, β-Catenin, Stem cell-like properties.

Lung cancer is the leading cause of cancer-related death in the developed world.1 Non–small-cell lung cancer (NSCLC) accounts for approximately 80% of the lung cancers and the prognosis of advanced NSCLC remains very poor despite advances in the treatment.2

One promising treatment strategy involves the further subdivision of NSCLC into clinically relevant molecular subsets, according to a classification schema based on specific so-called oncogenic driver mutations. These mutations occur in genes that encode signal proteins crucial for cellular proliferation and survival. Thus, cancer might rely on the expression of these single-mutant oncogenes for survival. This concept is also called oncogene addiction.3 The epidermal growth factor receptor (EGFR) is recognized as an important molecular target in cancer therapy,4 and somatic activating mutations of the EGFR gene (EGFR mutations) are one type of oncogenic driver mutation in NSCLC.5 Patients with NSCLC with EGFR mutations generally respond to EGFR tyrosine kinase inhibitors (EGFR-TKIs).6–11 Although these patients respond dramatically to treatment, the majority eventually undergo disease progression,12 and the threonine-to-methionine mutation in codon 790 (T790M) in exon 20 of the EGFR gene is the most common type of acquired resistance mutation.13–15

β-Catenin is a key component of the Wnt/β-Catenin signal and is an important oncogene that is involved in the pathogenesis and progression of malignant tumors including lung cancer.16–18 Numerous studies indicate that the Wnt/β-Catenin signal analogously contributes to cancer progression through the maintenance of highly tumorigenic subpopulations of cancer cells called cancer stem cells.19–22 A previous article has reported that excessive activation of the Wnt/β-Catenin signal exists in approximately 50% of the human NSCLC cell lines.23 Importantly, β-Catenin also interacts closely with the EGFR, phosphoinositide 3-kinase (PI3K)/murine thymoma viral oncogene (Akt) and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) signals,24 and a
positive correlation exists between the activated EGFR mutations and the nuclear accumulation of β-Catenin in NSCLC.\textsuperscript{23} Thus, β-Catenin may affect the efficacy of EGFR-TKI therapy by bypassing the stimulation of EGFR or its downstream signaling.\textsuperscript{26-28} However, the influence of the β-Catenin signal in EGFR-mutated NSCLC carrying the T790M mutation has not been previously reported. Hence, this subject was investigated in the present study.

**MATERIALS AND METHODS**

**Cell Culture**

The HEK293 cell line (human embryonic kidney cell line) was maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) (GIBCO BRL, Grand Island, NY). The A549 (wild-type EGFR gene), H1299 (wild-type EGFR gene), PC-9 (EGFR exon 19 deletion), and TACE684 (an ALK inhibitor) were purchased from Selleck Chemicals (Houston, TX). XAV939 (a β-Catenin inhibitor) and cisplatin (cis-diamminedichloroplatinum [CDDP]) were purchased from Sigma-Aldrich. For the in vitro studies, stock solutions were prepared in dimethyl sulfoxide. For the in vivo studies, BIBW2992 was suspended in a vehicle (0.5% methylcellulose [w/v] in sterile water) for oral administration to mice bearing xenograft tumors.

**Ligands and Reagents**

EGF, basic fibroblast growth factor (bFGF), and Wnt3a were purchased from R&D Systems (Minneapolis, MN). AG1478 (a reversible EGFR-TKI) was purchased from Wako (Tokyo, Japan). BIBW2992 (an irreversible EGFR-TKI) and TACE684 (an ALK inhibitor) were purchased from Selleck Chemicals (Houston, TX). XAV939 (a β-Catenin inhibitor) and cisplatin (cis-diamminedichloroplatinum [CDDP]) were purchased from Sigma-Aldrich. For the in vitro studies, stock solutions were prepared in dimethyl sulfoxide. For the in vivo studies, BIBW2992 was suspended in a vehicle (0.5% methylcellulose [w/v] in sterile water) for oral administration to mice bearing xenograft tumors.

**Growth Inhibition Assay In Vitro**

The growth-inhibitory effects of XAV939, CDDP, AG1478, BIBW2992, and TACE684 were examined using a 3, 4, 5-dimethyl-2H-tetrazolium bromide assay (MTT; Sigma-Aldrich), as described previously.\textsuperscript{29}

**Plasmid Construction, Viral Production, and Stable Transfectants**

A short hairpin RNA (shRNA)-targeting CTNNB1 gene was constructed using oligonucleotides encoding small interfering RNA directed against the CTNNB1 gene and a nonspecific target as follows: CTCAGATGGTGTCGTCTTAT for CTNNB1 shRNA (shRNA-CTNNB1), and TTCTGGAATACGCTCGTTG for control shRNA (shRNA-scramble). The oligonucleotides were cloned into an RNAi-Ready pSIREN-RetroQZsGreen vector (Clontech, Palo Alto, CA). The viral production and stable transfectant methods that were used have been previously described.\textsuperscript{30} The viral vector and stable viral transfectant cell lines were designated as pSIREN-CTNNB1, pSIREN-Scr, H1975/sh-CTNNB1, and H1975/sh-Scr, respectively.

**Antibody**

Antibodies specific for EGFR, phospho-EGFR, β-Catenin, cluster of differentiation (CD) 44, cyclin D1, cellular myelocytomatosis oncogene (cMYC), poly ADP-ribose polymerase (PARP), cleaved PARP, caspase 3, and β-actin were obtained from Cell Signaling (Beverly, MA). An antibody specific for active β-Catenin was obtained from Millipore (Bedford, MA).

**Western Blot Analysis**

A Western blot analysis was performed as described previously.\textsuperscript{31} Briefly, subconfluent cells were washed with cold phosphate-buffered saline (PBS) and harvested with Lysis A buffer containing 1% Triton X-100, 20 mM Tris-HCl (pH 7.0), 5 mM ethylenediaminetraacetic acid, 50 mM sodium chloride, 10 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate and a protease inhibitor mix, Complete (Roche Diagnostics, Basel, Switzerland). Whole-cell lysates were separated using sodium dodecyl sulfate-PAGE and were blotted onto a polyvinylidene fluoride membrane. After blocking with 3% bovine serum albumin in a tris-buffered saline buffer (pH 8.0) with 0.1% Tween-20, the membrane was probed with the primary antibody. After rinsing twice with tris-buffered saline buffer, the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody and washed, followed by visualization using an enhanced chemiluminescence (ECL) detection system (GE Healthcare, Buckinghamshire, UK) and LAS-3000 (Fujifilm, Tokyo, Japan). When the influence of the ligands was evaluated, the cultured medium was replaced with PBS-free medium 6 hours before exposure to the ligands. An inhibitor, when used, was added 30 minutes before the ligand stimulation.

**Sphere Formation Assay**

A total of 30,000 of cells were plated in six-well plates with a low-attachment surface and were then cultured in serum-free DMEM/F12 (Invitrogen, Carlsbad, CA) supplemented with 20 ng/ml EGF and 10 ng/ml bFGF in the presence of inhibitors for 48 hours (XAV939, 1 μM; BIBW2992, 0.05 μM; or a combination of both). The number of spheres exceeding 20 μm in size was counted for each well under a microscope. The experiment was performed in triplicates.

**Cell Cycle Distribution Analysis**

The cell cycle analyses were performed as described previously.\textsuperscript{32} Briefly, the cell line was exposed to the inhibitors (XAV939, 1 μM; BIBW2992, 0.05 μM; or a combination of both) for 48 hours. The cells were harvested by trypsinization and fixed with cold 70% ethanol at 4°C for 30 minutes. Then, the cells were washed twice with PBS and were stained using propidium iodide (PI)/RNase Staining Buffer (BD Biosciences, San Jose, CA). The viral production and stable transfectant methods that were used have been previously described.\textsuperscript{30} The viral vector and stable viral transfectant cell lines were designated as pSIREN-CTNNB1, pSIREN-Scr, H1975/sh-CTNNB1, and H1975/sh-Scr, respectively.
CA) at room temperature for 15 minutes. The cells were analyzed using a flow cytometer (BD FACSCalibur; BD Biosciences), and the cell cycle analysis was performed using ModFit LT software. The experiment was performed in triplicates.

**Annexin V Binding Apoptosis Analysis**

The Annexin V binding apoptosis analyses were performed as described previously. Briefly, the cells were exposed to the inhibitors (XAV939, 1 μM; BIBW2992, 0.05 μM; or a combination of both) for 48 hours, then the binding of Annexin V and PI to the cells was measured using a fluorescein isothiocyanate (FITC) Annexin V Apoptosis Detection Kit I (BD Biosciences) according to the manufacturer’s instructions. The cells were harvested using accutase and were stained with FITC Annexin V and PI at room temperature for 15 minutes. The cells were then analyzed using a flow cytometer and Cell Quest software (BD Biosciences). The experiment was performed in triplicates.

**Xenograft Studies**

Nude mice (Bagg albino [BALB]/c nu/nu; 6-week-old females; CLEA Japan, Tokyo, 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 the effect of BIBW2992, a suspension of 5 × 10^6 H1975 transfectant cells (in 50 μl PBS) with 50 μl of Matrigel were subcutaneously inoculated into nude mice (n = 7). The treatment was initiated when the tumors in each group of mice achieved an average volume of approximately 100 mm³. In the treatment groups, BIBW2992 (15 mg/kg) was administered by oral gavage daily for 28 days; the control animals received 0.5% methylcellulose as the vehicle. The tumor volume was calculated as the length × width^2 × 0.5. The tumor formation and volume were assessed every 2 to 3 days. At the end of the experiment, the mice were killed and the xenografts were resected, fixed in 10% buffered formalin for 6 to 10 hours, and processed for histologic analysis. The method was described previously.

**Immunohistochemical Analysis**

The cancer cells undergoing apoptosis were detected using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method and the in situ apoptosis detection TUNEL kit (TaKaRa, Shiga, Japan) according to the manufacturer’s recommended protocol, as described previously.

**Statistical Analysis**

Continuous variables were analyzed using the Student’s t test, and the results were expressed as the average and SDs. The statistical analyses were two-tailed and were performed using Microsoft Excel (Microsoft, Redmond, WA). A p value of less than 0.05 was considered statistically significant.

**RESULTS**

**Expression of β-Catenin and Sensitivity to XAV939 in Lung Cancer Cell Lines**

To examine the expression of β-Catenin in lung cancer cell lines, we performed a Western blot analysis. The cell lines with the wild-type EGFR gene (A549 and H1299) had a low expression of β-Catenin and active β-Catenin (active form of β-Catenin, dephosphorylated on Ser37 or Thr41). In contrast, the EGFR-mutated NSCLC cell lines (PC-9, PC-9/ZD, 11_18, and H1975) had a high expression of β-Catenin and active β-Catenin (Fig. 1A). This result supports the findings of a previous study showing that a positive correlation exists between activated EGFR mutations and the nuclear accumulation of β-Catenin in NSCLC. Despite the high expression, the lung cancer cell lines, including the EGFR-mutated cell lines, were not very sensitive to XAV939 (Fig. 1B).

**XAV939 Enhanced the Sensitivity to EGFR-TKIs in EGFR-Mutated NSCLC Cell Lines**

Next, we examined the synergistic effect of XAV939 using several reagents. XAV939 did not influence the sensitivity to the cytotoxic agent CDDP in either the A549 (wild-type

![Image](https://example.com/image.png)

**FIGURE 1.** Expression of β-Catenin and sensitivity to its inhibitor, XAV939. A, expression of β-Catenin and active β-Catenin. The expression was determined using a Western blot analysis, and β-actin was used as an internal control. EGFR-mutated NSCLC cell lines (PC-9, 11_18, PC-9/ZD, and H1975) had high expression levels, compared with cell lines with the wild-type EGFR gene (HEK293, A549, and H1299), (B), sensitivities to XAV939. The growth-inhibitory effect was examined using an MTT assay. The experiment was performed in triplicate. The 50% inhibitory concentrations of the A549, PC-9, PC-9/ZD, and H1975 cell lines were 164, 100, 27, and 57 μM, respectively. The cell lines, including the EGFR-mutated NSCLC cell lines, were not very sensitive to the inhibitor despite their high expression levels. Lines, mean of independent triplicate experiments. NSCLC, non–small-cell lung cancer.
**FIGURE 2.** Synergistic effect of XAV939 in NSCLC cell lines. The growth-inhibitory effect was examined using an MTT assay. The experiment was performed in triplicate, and dimethyl sulfoxide (DMSO) was used for the control. To evaluate the synergistic effect, the concentrations of XAV939 (a β-Catenin inhibitor), CDDP, AG1478 (a reversible EGFR-TKI), BIBW2992 (an irreversible EGFR-TKI), and TAE684 (an ALK inhibitor) that were used were 1, 300, 0.01, and 0.02 μM, respectively. A, synergistic effect in the A549 cell line (wild-type *EGFR* gene). XAV939 alone did not inhibit the proliferation and did not enhance the inhibitory effects of CDDP and AG1478. In other words, XAV939 did not enhance the sensitivities to either CDDP or AG1478 in the A549 cell line, (B), synergistic effect in the PC-9 cell line (*EGFR* exon 19 deletion). XAV939 alone did not inhibit the proliferation and did not enhance the sensitivity to CDDP, similar to the results obtained in the A549 cell line, but XAV939 greatly inhibited the proliferation when used in combination with AG1478, compared with AG1478 alone. In other words, XAV939 enhanced the sensitivity to AG1478. In the 11_18 cell line (*EGFR* exon 21 L858R), a similar synergistic effect was observed (data not shown), (C), synergistic effect in the PC-9/ZD cell line (*EGFR* exon 19 deletion and exon 20 T790M). XAV939 alone did not inhibit the proliferation, and the inhibitory effect was not enhanced when XAV939 was used in combination with AG1478. In contrast, XAV939 greatly inhibited the proliferation when used in combination with BIBW2992, compared with BIBW2992 alone. In other words, XAV939 enhanced the sensitivity to BIBW2992, (D), synergistic effect in the H1975 cell line (*EGFR* exon 21 L858R and exon 20 T790M). XAV939 alone slightly inhibited the proliferation, but the inhibitory effect was not enhanced when XAV939 was used in combination with AG1478. In contrast, XAV939 greatly inhibited the proliferation when used in combination with BIBW2992, compared with BIBW2992 alone. In other words, XAV939 enhanced the sensitivity to BIBW2992, (E), synergistic effect in the H3122 cell line (wild-type *EGFR* gene and ALK rearrangement). XAV939 alone slightly inhibited the proliferation, but the inhibitory effect was not enhanced when used in combination with either AG1478 or TAE684. AG, AG1478; XAV, XAV939; BIBW, BIBW2992; TAE, TAE684; Columns, mean of independent triplicate experiments; bars, SD; n.s., not significant; *p < 0.05.

*EGFR* gene) or the PC-9 (*EGFR* exon 19 deletion) cell lines (Fig. 2A, B). XAV939 also did not influence the sensitivity to a reversible EGFR-TKI, AG1478, in the A549 and H3122 (wild-type *EGFR* gene) cell lines (Fig. 2A, E), whereas it enhanced the sensitivity to AG1478 in the PC-9 and the 11_18 (*EGFR* exon 21 L858R) cell lines (Fig. 2B). In the PC-9/ZD (*EGFR* exon 19 deletion and exon 20 T790M) and the H1975 cell lines (*EGFR* exon 21 L858R and exon 20 T790M), however, XAV939 did not enhance the sensitivity to AG1478 (Fig. 2C, D). In contrast, XAV939 enhanced
the sensitivity to an irreversible EGFR-TKI, BIBW2992, in the PC-9/ZD and the H1975 cell lines (Fig. 2C, D). Finally, in the H3122 cell line (wild-type EGFR gene and ALK rearrangement [another driver mutation in NSCLC]), XAV939 did not enhance the sensitivity to the ALK inhibitor TAE684 (Fig. 2E). These results indicate that β-Catenin inhibition affects the EGFR signal, in particular.

Interaction between EGFR and β-Catenin Signal

Since the effect of β-Catenin inhibition was observed in the EGFR-mutated NSCLC cell lines and with EGFR-TKIs, we investigated the EGFR signal using a Western blot analysis. In the PC-9 cell line, the phosphorylation of EGFR was not influenced by XAV939 alone, whereas it was decreased by AG1478 alone. Moreover, AG1478 decreased the expression of active β-Catenin, along with the decreased phosphorylation of EGFR. The combination of XAV939 and AG1478 strongly decreased the phosphorylation of EGFR and the expression of active β-Catenin in the H1975 cell line (EGFR exon 21 L858R and exon 20 T790M), AG1478 did not influence the phosphorylation of EGFR or the expression of active β-Catenin. Even when used in combination with XAV939, AG1478 did not influence the phosphorylation of EGFR, (B), influence of XAV939 and BIBW2992. In the PC-9/ZD and H1975 cell lines, BIBW2992 decreased the phosphorylation of EGFR and the expression of active β-Catenin. The combination of XAV939 and BIBW2992 strongly decreased the phosphorylation of EGFR and the expression of active β-Catenin, compared with AG1478 alone. In the H1975 cell line (EGFR exon 21 L858R and exon 20 T790M), AG1478 did not influence the phosphorylation of EGFR or the expression of active β-Catenin. Even when used in combination with XAV939, AG1478 did not influence the phosphorylation of EGFR, (B), influence of XAV939 and BIBW2992. In the PC-9/ZD and H1975 cell lines, BIBW2992 decreased the phosphorylation of EGFR and the expression of active β-Catenin. The combination of XAV939 and BIBW2992 strongly decreased the phosphorylation of EGFR and the expression of active β-Catenin, compared with AG1478 alone. Even when used in combination with XAV939, AG1478 did not influence the phosphorylation of EGFR, (B), influence of XAV939 and BIBW2992. In the PC-9/ZD and H1975 cell lines, BIBW2992 decreased the phosphorylation of EGFR and the expression of active β-Catenin. The combination of XAV939 and BIBW2992 strongly decreased the phosphorylation of EGFR and the expression of active β-Catenin, compared with AG1478 alone. Even when used in combination with XAV939, AG1478 did not influence the phosphorylation of EGFR, (B), influence of XAV939 and BIBW2992. In the PC-9/ZD and H1975 cell lines, BIBW2992 decreased the phosphorylation of EGFR and the expression of active β-Catenin. The combination of XAV939 and BIBW2992 strongly decreased the phosphorylation of EGFR and the expression of active β-Catenin, compared with AG1478 alone. Even when used in combination with XAV939, AG1478 did not influence the phosphorylation of EGFR, (B), influence of XAV939 and BIBW2992. In the PC-9/ZD and H1975 cell lines, BIBW2992 decreased the phosphorylation of EGFR and the expression of active β-Catenin. The combination of XAV939 and BIBW2992 strongly decreased the phosphorylation of EGFR and the expression of active β-Catenin, compared with AG1478 alone. Even when used in combination with XAV939, AG1478 did not influence the phosphorylation of EGFR, (B), influence of XAV939 and BIBW2992. In the PC-9/ZD and H1975 cell lines, BIBW2992 decreased the phosphorylation of EGFR and the expression of active β-Catenin. The combination of XAV939 and BIBW2992 strongly decreased the phosphorylation of EGFR and the expression of active β-Catenin, compared with AG1478 alone. Even when used in combination with XAV939, AG1478 did not influence the phosphorylation of EGFR, (B), influence of XAV939 and BIBW2992.
XAV939 alone did not influence the phosphorylation of EGFR in the cell lines with phosphorylated EGFR, but it strongly decreased phosphorylation when used in combination with EGFR-TKI, compared with EGFR-TKI alone. Then, to examine whether β-Catenin influences the EGFR signal in cell line without phosphorylated EGFR, the phosphorylation of EGFR after Wnt3a stimulation was investigated. In the A549 cell line (unphosphorylated EGFR), the phosphorylation of EGFR after Wnt3a stimulation increased slightly, but the effect was cancelled by XAV939 (Fig. 3C). In the PC-9 cell line (phosphorylated EGFR), Wnt3a did not increase the phosphorylation of EGFR but it did increase the phosphorylation that had been decreased by AG1478 (Fig. 3D). In addition, the increased phosphorylation was inhibited by XAV939 (Fig. 3D). These results suggest that the EGFR and β-Catenin signals interact with each other, as previously reported (Fig. 4).24

**Association of the β-Catenin Signal with Stem Cell-Like Properties in the H1975 Cell Line**

Because the β-Catenin signal is associated with cancer stem cells,19–22 the influences of inhibitors on sphere formation, which is a representative stem cell assay, were investigated in the H1975 cell line. XAV939 or BIBW2992 alone inhibited sphere formation (Fig. 5A). Furthermore, the combination of both the agents greatly inhibited sphere formation, compared with the effects of each agent alone (Fig. 5A). In the spheres of the H1975 cell line, downstream expressions related to stem cell-like properties (CD44, cMYC, and cyclin D1) were also decreased by XAV939 or BIBW2992 alone and were greatly decreased by the combination of both the agents (Fig. 5B).

**Combination of XAV939 and BIBW2992 Decreased S Phase and Enhanced Apoptosis in the H1975 Cell Line**

Next, we analyzed the cell cycle distribution and Annexin V binding apoptosis of the H1975 cell line after exposure to XAV939 and BIBW2992. Each of the single reagents decreased S phase and induced apoptosis, while the combination of these reagents greatly decreased the S phase and induced apoptosis (Fig. 5C, D). Western blot analyses for apoptosis-related molecules revealed that the combination of these agents also increased the levels of cleaved PARP and cleaved caspase 3 (Fig. 5E).

**β-Catenin Knockdown Enhanced the Sensitivity to BIBW2992 in the H1975 Cell Line In Vivo**

To perform a xenograft study, we created a β-Catenin or non-specific target knockdown H1975 cell line using a shRNA-targeting CTNNB1 gene or a nonspecific target (H1975/sh-CTNNB1 and H1975/sh-Scr, respectively) (Fig. 6A). BIBW2992 exhibited an enhanced effect in H1975/sh-CTNNB1, compared with H1975/sh-Scr (CTNNB1: 205.6±40.87 mm³ versus Scr: 342.2±72.05 mm³; p = 0.035, on day 29) (Fig. 6B). TUNEL staining of the tumor specimens showed that apoptosis was increased in H1975/sh-CTNNB1 cells (Fig. 6C).

**DISCUSSION**

**EGFR mutations occur in 30% of the patients with NSCLC who are of East Asian ethnicity compared with 8% of the patients of other ethnicities.33 Despite dramatic responses to treatment with EGFR-TKIs, patients with NSCLC who harbor EGFR mutations will eventually experience disease progression as a result of the inevitable development of resistance mechanisms, particularly, the T790M mutation, which is found in more than 50% of the patients who are treated with EGFR-TKIs.13–15 Irreversible ErbB family blockers are called second generation EGFR-TKIs and include BIBW2992, the preclinical activity of which has been shown to include EGFR-mutated cell lines carrying the T790M mutation.34,35 However, the clinical trials of second generation EGFR-TKIs in patients with acquired resistance have demonstrated disappointing results.36–38 Therefore, there is an increasing need to develop new molecular targeted agents. In the present study, we found**

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**FIGURE 4.** Diagram for the proposed effects of β-Catenin on EGFR mutations. A, normal state. β-Catenin closely interacts with EGFR, (B), EGFR-mutated state. The activated EGFR activates the β-Catenin signal. However, β-Catenin signal has a minimal influence on the EGFR signal, which has already been activated, (C), EGFR-mutated state with EGFR-TKI. EGFR-TKI inhibits the activated EGFR signal, resulting in the suppression of the β-Catenin signal. However, the remaining β-Catenin signal may activate the EGFR signal that had been inhibited by the EGFR-TKI, (D), EGFR-mutated state with XAV939 and EGFR-TKI. Both the β-Catenin signal and the EGFR signal are inhibited, resulting in the inhibition of the interaction. Therefore, the proliferation, survival, and stem cell-like properties are greatly suppressed.
that the β-Catenin signal was activated in EGFR-mutated NSCLC cell lines, as previously reported, and was associated with stem cell-like properties. Furthermore, the inhibition of this signal enhanced the antitumor effect of BIBW2992 against EGFR-mutated NSCLC carrying the T790M mutation. Although some previous studies have revealed that β-Catenin can affect the efficacy of EGFR-TKI therapy, this is the first report to show that the inhibition of the β-Catenin signal enhances the antitumor effect of BIBW2992 for EGFR-mutated NSCLC carrying the T790M mutation.

An interaction between EGFR and the β-Catenin signal has been reported, and our experiments have demonstrated that EGFR-TKI alone inhibits the β-Catenin signal and that the combination of XAV939 and EGFR-TKI greatly inhibits both the β-Catenin signal and the EGFR signal, compared with XAV939 or EGFR-TKI alone. These results suggest that this synergistic effect comes not only from inhibiting the bypassing role of the β-Catenin signal, but also by inhibiting the interaction (Fig. 4). In an EGFR-mutated NSCLC cell line, the β-Catenin signal does not activate the EGFR signal, which has been already activated, and XAV939 alone does not inhibit the already activated EGFR signal. In general, reversible EGFR-TKIs cannot inhibit the EGFR signal in cells with the acquired resistance T790M mutation because of

FIGURE 5. Stem cell-like properties, cell cycle, and apoptosis in the H1975 cell line (EGFR exon 21 L858R and exon 20 T790M). The concentrations of XAV939 (a β-Catenin inhibitor) and BIBW2992 (an irreversible EGFR-TKI) that were used were 1 and 0.05 μM, respectively. A, sphere formation of H1975 cell line. A total of 30,000 of cells were plated in six-well plates with a low attachment surface, and the cells were cultured in serum-free DMEM/F12 supplemented with 20 ng/ml EGF and 10 ng/ml bFGF in the presence of inhibitors. The number of spheres exceeding 20 μm in size was counted for each well under a microscope. The experiment was performed in triplicates, and DMSO was used for the control. Treatment with XAV939 or BIBW2992 reduced the size and number of spheres (p = 0.042* and 0.043* respectively). The combination of these two agents further decreased the size and the number of spheres, compared with XAV939 or BIBW2992 alone (p = 0.046* and 0.013* respectively). Scale bar, 20 μm. Columns, mean of independent triplicate experiments; bars, SD; *p < 0.05, (B), Western blot analyses of the β-Catenin signal and its downstream expression in the spheres of H1975 cell line. β-actin was used as an internal control. The cell line was exposed to the inhibitors for 48 hours before the Western blot analyses. XAV939 or BIBW2992 alone suppressed the downstream expression (CD44, cMYC, and cyclin D1), and the combination of both the agents greatly suppressed the expression, (C), cell cycle distribution in the H1975 cell line. The cell line was exposed to the inhibitors for 48 hours. The cells were harvested, fixed, and stained using PI/RNase Staining Buffer. The cells were analyzed using a flow cytometer. The experiment was performed in triplicates, and DMSO was used for the control. XAV939 or BIBW2992 alone decreased S phase (p = 0.0024* and 0.0031*, respectively), and the combination of both the agents greatly decreased S phase, compared with XAV939 or BIBW2992 alone (p = 0.0029* and 0.0020*, respectively). Columns, mean of independent triplicate experiments; bars, SD; *p < 0.05, (D), annexin V binding apoptosis analyses. The cells were exposed to the inhibitors (XAV939, 1 μM; BIBW2992, 0.05 μM; or a combination of both) for 48 hours and were then harvested and stained with FITC Annexin V and PI. The cells were analyzed using a flow cytometer. The experiment was performed in triplicates, and DMSO was used for the control. XAV939 or BIBW2992 alone induced apoptosis (p = 0.011* and 0.011*, respectively), and the combination of both the agents further induced apoptosis, compared with XAV939 or BIBW2992 alone (p = 0.018* and 0.015*, respectively). Columns, mean of independent triplicate experiments; bars, SD; *p < 0.05, (E), Western blot analyses for apoptosis-related molecules in the H1975 cell line. β-actin was used as an internal control. XAV939 or BIBW2992 alone increased the expression of cleaved PARP and cleaved caspase 3, and the combination of agents further increased these molecules, compared with XAV939 or BIBW2992 alone. C-PARP, cleaved PARP, C-caspase 3, cleaved caspase 3.
their structure and affinity for ATP. Therefore, in the PC-9/ ZD and H1975 cell lines, which harbor the acquired resistance T790M mutation, AG1478 (a reversible EGFR-TKI) did not inhibit the EGFR signal and XAV939 did not inhibit the already activated EGFR signal, resulting in no synergistic effect. In contrast, BIBW2992 (an irreversible EGFR-TKI) inhibited the EGFR signal and exhibited a synergistic effect in the PC-9/ZD and H1975 cell lines. Therefore, in EGFR-mutated NSCLC carrying the T790M mutation, β-Catenin could become a novel therapeutic target when used in combination with irreversible EGFR-TKIs, such as BIBW2992, but not with reversible EGFR-TKIs. In addition, several recent studies have demonstrated that third-generation EGFR-TKIs, which are mutant-selective inhibitors, can overcome T790M-mediated resistance. Therefore, β-Catenin might also become a potential therapeutic target in treatments combined with the use of these third-generation EGFR-TKIs.

Numerous studies have reported an association between cancer stem cells and chemotherapy resistance, and several studies have examined the association between stem cell-like properties and EGFR-TKI resistance. Numerous studies indicate that the Wnt/β-Catenin pathway contributes to cancer stem cells. In the present study, we showed that the inhibition of the β-Catenin signal contributed to the inhibition of sphere formation, that is, stem cell-like properties, in the H1975 cell line, suppressing downstream CD44, cMYC, and cyclin D1. Although another previous study in which cyclin D1 was suppressed by EGFR-TKIs in the H1975 cell line has been reported, our study is the first report to show that not only cyclin D1, but also the β-Catenin signal is associated with the antitumor effect of EGFR-TKIs in the H1975 cell line. A recent study has shown that first-line treatment with BIBW2992 significantly prolonged survival, compared with first-line platinum based chemotherapy, in patients with EGFR-mutated NSCLC, especially carrying the exon 19 deletion, suggesting that NSCLC carrying the EGFR exon 19 deletion might be more sensitive to BIBW2992 than to other EGFR-TKIs. Furthermore, these data raise the possibility that platinum based chemotherapy may make NSCLC carrying EGFR mutations less sensitive to second-line or later treatments with EGFR-TKIs. Reportedly, platinum-based chemotherapy induces stem cell-like properties via the β-Catenin signal. Thus, the inhibition of β-Catenin may enable such resistance to be overcome.

In conclusion, we found that the inhibition of the β-Catenin signal enhanced the sensitivity to EGFR-TKIs in EGFR-mutated NSCLC, an effect that was seen using an irreversible EGFR-TKI and EGFR-mutated NSCLC carrying the T790M mutation. This synergistic effect arose from the inhibition of the interaction between EGFR and the β-Catenin signal. In addition, the inhibition of β-Catenin signal contributed to the suppression of the stem cell-like properties, which are also related to EGFR-TKI resistance. Our findings indicate that β-Catenin could become a novel therapeutic target for EGFR-mutated NSCLC carrying the T790M mutation.

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FIGURE 6. Xenograft study. A, Western blot analyses of H1975 transfectant cell lines. β-actin was used as an internal control. CTNNB1-knockdown was confirmed in the cell lines, (B), xenograft study. A total of 5 x 10^6 H1975 transfectant cells (in 50 μl PBS) with 50 μl of Matrigel were subcutaneously inoculated into nude mice (n = 7). Treatment was initiated when the tumors in each group of mice achieved an average volume of approximately 100 mm^3. In the treatment groups, BIBW2992 (15 mg/kg) was administered by oral gavage daily for 28 days; the control animals received 0.5% methylcellulose as a vehicle. The tumor formation and volume were assessed every 2 to 3 days. After the initiation of the treatment, the H1975/sh-CTNNB1 cell line produced a significantly smaller tumor volume than the H1975/sh-Scr cell line on day 29 (sh-Scr: 342 ± 72 mm^3 versus sh-CTNNB1: 206 ± 41 mm^3; p = 0.035*), whereas, in the control group, there was no significant difference between the H1975/sh-CTNNB1 cell line and the H1975/sh-Scr cell line (sh-Scr: 487 ± 107 mm^3 versus sh-CTNNB1: 464 ± 99 mm^3; p = 0.84). Lines, mean of seven tumors; error bars, SD; n.s., not significant; *p < 0.05, (C), TUNEL staining of the xenograft samples. TUNEL staining of the tumor specimens showed that apoptosis was increased in the H1975/sh-CTNNB1 cells. PBS, phosphate-buffered saline.
REFERENCES


