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RESEARCH ARTICLE

Moulid Hidayat, et al.: Role of FBXW7 in gefitinib-resistant lung cancer stem cells

Role of FBXW7 in the quiescence of gefitinib-resistant lung cancer stem cells in EGFR-mutant non-small cell lung cancer


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ABSTRACT

Several recent studies suggest that cancer stem cells (CSCs) are involved in intrinsic resistance to cancer treatment. Maintenance of quiescence is crucial for establishing resistance of CSCs to cancer therapeutics. F-box/WD repeat-containing protein 7 (FBXW7) is a ubiquitin ligase that regulates quiescence by targeting the c-MYC protein for ubiquitination. We previously reported that gefitinib-resistant persisters (GRPs) in EGFR-mutant non-small cell lung cancer (NSCLC) cells highly expressed octamer-binding transcription factor 4 (Oct-4) as well as the lung CSC marker CD133, and they exhibited distinctive features of the CSC phenotype. However, the role of FBXW7 in lung CSCs and their resistance to epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitors in NSCLC is not fully understood. In this study, we developed GRPs from the two NSCLC cell lines PC9 and HCC827, which express an EGFR exon 19 deletion mutation, by treatment with a high concentration of gefitinib. The GRPs from both PC9 and HCC827 cells expressed high levels of CD133 and FBXW7, but low levels of c-MYC. Cell cycle analysis demonstrated that the majority of GRPs existed in the G0/G1 phase. Knockdown of the FBXW7 gene significantly reduced the cell number of CD133-positive GRPs and reversed the cell population in the G0/G1-phase. We also found that FBXW7 expression in CD133-positive cells was increased and c-MYC expression was decreased in gefitinib-resistant tumors of PC9 cells in mice and in 9 out of 14 tumor specimens from EGFR-mutant NSCLC patients with acquired resistance to gefitinib. These findings suggest that FBXW7 plays a pivotal role in the maintenance of quiescence in gefitinib-resistant lung CSCs in EGFR
mutation-positive NSCLC.

**KEYWORDS:** FBXW7; quiescence; cancer stem cells; gefitinib resistance; NSCLC
INTRODUCTION

Cancer stem cells (CSCs), also known as tumor-initiating cells and stem-like cancer cells, are thought to constitute a minor subpopulation of cancer cells [1]. Accumulating evidence indicates that CSCs persist after cancer treatments such as chemotherapy, radiotherapy, and molecularly targeted therapy [2]. Recent studies revealed that CSCs are maintained in a non-proliferative state, referred to as quiescence, dormancy, or G0 phase, which is thought to render CSCs resistant to various cancer therapeutics [3].

Advanced non-small cell lung cancer (NSCLC) is the leading cause of cancer-related deaths globally [4]. Somatic mutations in the epidermal growth factor receptor (EGFR) gene, such as an in-frame deletion mutation in exon 19 and the point mutation L858R, are associated with a favorable response to the EGFR tyrosine kinase inhibitors (EGFR-TKIs) gefitinib and erlotinib [5]. However, primary or acquired resistance to EGFR-TKIs is a major obstacle to improving the prognosis of EGFR mutation-positive NSCLC patients. The mechanisms of resistance identified to date include secondary mutation of EGFR T790M, amplification of MET, HGF overexpression, HER2 amplification or mutation, conversion to small cell lung cancer, and epithelial mesenchymal transition (EMT) [6, 7]. However, the mechanisms responsible for resistance to EGFR-TKIs are not well understood.

FBXW7 (F-box and WD40 repeat domain-containing 7), also known as FBW7, sel-10, hCDC4, or hAgo, is a substrate-recognition subunit of the SCF (Skp1-Cull-F-box protein)-type ubiquitin ligase
complex [8, 9]. Several studies demonstrated that FBXW7 is involved in quiescence by degradation of c-MYC protein. It has been reported that FBXW7 plays an important role in the maintenance of quiescence in leukemia-initiating cells (LICs) by reducing the level of c-MYC protein. Furthermore, abrogation of quiescence in LICs by FBXW7 ablation increased sensitivity to the tyrosine kinase inhibitor imatinib [10]. Thus, targeting quiescence might be a promising strategy for effective control of CSCs.

We previously reported that gefitinib-resistant persisters (GRPs) in EGFR-mutant NSCLC cells showed elevated expression of stem cell genes including CD133, OCT4, SOX2, NANOG, CXCR4, and ALDH1A1, and they showed characteristic features of CSCs [11, 12]. In this study, we examined whether FBXW7 plays a crucial role in the maintenance of quiescence in gefitinib-resistant CSCs by using an in vitro as well as in vivo GRPs model with stem cell features. We also evaluated the cell cycle status by introducing a FUCCI (fluorescence ubiquitination cell cycle indicator)-expressing plasmid into GRPs. The biological role of FBXW7 for the maintenance of quiescence in gefitinib-resistant lung CSCs in EGFR-mutant NSCLC is discussed.

MATERIALS AND METHODS

Cell culture and reagents

In this study we used two NSCLC cell lines, PC9 and HCC827, which harbor deletion mutations of EGFR exon 19 (ΔE746-A750) as previously depicted [13]. The reagents and condition of the culture
are explained in the online supplement of Materials and Methods.

Quantitative real-time PCR

The quantitative polymerase chain reaction (qPCR) conditions and sequences of the primers applied for transcript detection are explained in the online supplement of Materials and Methods.

RNA interference

Short interfering RNAs (siRNAs) inhibiting \textit{FBXW7} (Stealth Select RNAi siRNA), a negative control, and lipofectamine RNAiMAX were purchased from Invitrogen (Carlsbad, CA, USA). The lipofectamine RNAiMAX and RNAi duplex were mixed in Opti-MEM®I (Gibco). The details of this procedure are explained in the online supplement of Materials and Methods.

Immunofluorescence

Cells were cultured either on Lab-Tek chamber II slides (Nunc, Rochester, NY, USA), or on 35 mm glass-bottom dishes (Greiner Bio-One, Frickenhausen, Germany) with 1 μM gefitinib for 72 hours, and immunofluorescence of \textit{FBXW7}, c-MYC, and CD133 were conducted as illustrated in the online supplement of Materials and Methods. The number of \textit{FBXW7}, c-MYC, and CD133-positive cells were counted; the ratio of positive cells to the total cell number was calculated in five fields for each experiment.
FUCCI (fluorescence ubiquitination cell cycle indicator)

pFucci-S/G2/M green and pFucci-G1 orange plasmids were purchased from MBL (Nagoya, Japan). 

*Fucci-S/G2/M green (mKO2-hCdt1)* and *Fucci-G1 orange (mAG-hGem)* were amplified by polymerase chain reaction (PCR) using LA Taq DNA Polymerase (TaKaRa Bio, Kyoto, Japan), and they were linked in-frame by a T2A sequence [14]. Then, the *Fucci-S/G2/M green-T2A-Fucci-G1 orange* fusion gene was cloned into the lentiviral vector CSII-CMV (kindly provided by Dr. Miyoshi, RIKEN BioResource Center, Tsukuba, Japan), and the resulting plasmid was designated as CSII-CMV-FUCCI-S/G2/M green-G1 orange. The plasmid of CSII-CMV-FUCCI-S/G2/M green-G1 orange was mixed with packaging plasmids and transfected into 293T cells (Invitrogen). Lentiviral infection was carried out as previously depicted [15]. FUCCI-expressing positive cells were used for further experiments.

Mice

The NOD/Shi-scid/IL-2Rnull (NOG) mice (seven-week-old, female) were obtained from the Central Institute for Experimental Animals (Kanagawa, Japan). The mice were lodged as illustrated in the online supplement of Materials and Methods.

Establishment of gefitinib-resistant tumors (GRTs) *in vivo*

PC9 cells (*1 × 10^5*) were inoculated into NOG mice, and when tumors reached the size of 75 mm²,
we started intraperitoneal injection to these mice with gefitinib. The tumors still remained after 14 days of gefitinib treatment, and we called these remaining tumors as gefitinib-resistant tumors (GRTs). Fourteen days after gefitinib administration, tumors were taken as illustrated in the online supplement of Materials and Methods.

**Immunohistochemistry**

In this study we used tumor specimens from NSCLC patients at Juntendo University Hospital before gefitinib treatment and after relapse with patients’ consent under a protocol that Institutional Review Board approved. Immunohistochemistry for FBXW7, c-MYC, and CD133 were carried out as depicted in the online supplement of Materials and Methods.

**Ethics**

All animal experiments were conducted in accordance with the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology of Japan (Notice No. 71, 2006) and were approved by the Committee for Animal Experimentation of Juntendo University with the Approval No. 240182.

**Statistical analysis**

Statistical analyses were performed in GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA). The
two-tailed Student’s *t*-test and ANOVA were used to compare values. Significant differences between the means were statistically regarded if $p < 0.05$.

RESULTS

Gefitinib-resistant persisters (GRPs) expressed high levels of FBXW7 and CD133 and low levels of c-MYC.

We developed GRPs from two NSCLC cell lines, PC9 and HCC827, harboring a sensitive *EGFR* mutation by exposing cells to a high concentration of gefitinib. After nine days, the vast majority of cells were dead but a small population of viable cells remained. We called these remaining cells “gefitinib-resistant persisters of PC9 and HCC827” (PC9-GRPs and HCC827-GRPs). We previously demonstrated using genomic DNA analysis that short tandem repeat (STR) portraits of the GRPs and parental cells were similar, and direct sequencing revealed that GRPs still harbored the *EGFR* exon 19 deletion mutation [11]. These results indicate that GRPs were not derived from contaminating cells. Furthermore, we also previously revealed that GRPs also did not harbor either the secondary mutation of *EGFR* T790M or amplification of *MET* [11].

We then investigated the expression level of FBXW7 and the lung CSC marker CD133, as well as c-MYC in parental cells and GRPs nine days after exposure to gefitinib. Expression levels of *FBXW7* and *CD133* mRNA were higher in GRPs of both PC9 and HCC827, as confirmed by qPCR analysis (Fig. 1A). We also evaluated the protein expression level of FBXW7 and c-MYC by
immunofluorescence in GRPs of PC9 and HCC827 cells after exposing cells to gefitinib for 72 hours. Immunofluorescence analysis showed increased expression of FBXW7 and loss of c-MYC expression in PC9-GRPs and HCC827-GRPs (Fig. 1B, C).

**Knockdown of FBXW7 decreased CD133-positive GRPs and reversed c-MYC expression in vitro.**

To investigate the role of FBXW7 in the maintenance of GRPs, FBXW7 was silenced by using two different siRNA (siRNA#1 and siRNA#2) in GRPs of PC9 and HCC827. The expression of FBXW7 was significantly suppressed by two different siRNAs in GRPs of PC9 and HCC827 cells (Fig. 2A, B and S1A, B). Furthermore, the reduction in FBXW7 expression significantly reversed c-MYC protein expression (Fig. 2C and S1C). We also examined CD133-positive GRPs after inhibition of FBXW7. CD133 mRNA expression and the number of CD133-positive GRPs were significantly decreased by using the two siRNAs targeting FBXW7 in the GRPs of PC9 cells and HCC827 cells (Fig. 2D, E and S1D, E). These results collectively suggest that FBXW7 regulates and maintains CD133-positive GRPs of PC9 and HCC827 cells and mediates degradation of c-MYC at the protein level.

**The majority of GRPs exist in G0/G1 phase, and FBXW7 is implicated in the quiescence of GRPs**

To examine the cell cycle status, we introduced a FUCCI-vector plasmid into PC9 cells and HCC827
cells to develop FUCCI-expressing PC9 cells (PC9-FUCCI) and FUCCI-expressing HCC827 cells (HCC827-FUCCI) using a lentiviral transfection procedure. Transfection efficiency was confirmed by the appearance of fluorescent color in both cell types using fluorescence microscopy. To clarify whether the GRPs were in a quiescent state, PC9-FUCCI cells and HCC827-FUCCI cells were treated with or without a high concentration of gefitinib (1 µM) for 72 hours and were subjected to cell cycle analysis. The cells in G0/G1 or S/G2/M phases appeared red and green, respectively [16].

To determine whether the same cells expressed CD133 and FBXW7 in GRPs, we conducted double immunofluorescence staining. We found that GRPs of PC9-FUCCI and GRPs of HCC827-FUCCI expressed both FBXW7 and CD133 in the quiescent state (G0/G1 phase) (Fig. S2). Cell cycle analysis demonstrated that the vast majority of cells exist in G0/G1 phase after gefitinib treatment as compared with untreated cells (Fig. 3A, S3A). To determine whether the upregulation of FBXW7 could maintain the quiescence of GRPs, we knocked down FBXW7 in PC9-FUCCI and HCC827-FUCCI using transfection of two specific siRNAs followed by gefitinib treatment for 72 hours. We found that most FBXW7-silenced cells were in S/G2/M phase, whereas control cells were still in G0/G1 phase (Fig. 3B and S3B). The number of GRPs was significantly decreased following knockdown of FBXW7 in FUCCI-expressing PC9 and HCC827 cells (Fig. 3C and S3C). Taken together, these findings suggest that GRPs largely exist in G0/G1 phase and that FBXW7 plays a pivotal role in the quiescence of GRPs.
FBXW7 expression in CD133-positive cells was increased and c-MYC expression was decreased in gefitinib resistant tumors (GRTs) *in vivo* as well as in tumor specimens from NSCLC patients with acquired resistance to gefitinib.

To further evaluate the expression of FBXW7 in the CD133-positive cells and c-MYC in the gefitinib-resistant tumors of NSCLC cells *in vivo*, we inoculated $1 \times 10^5$ PC9 cells into NOG mice. After the tumor size reached $75 \text{ mm}^3$, we injected 20 mg/kg of gefitinib to the mice or vehicle intraperitoneally. Following 14 to 17 days of gefitinib treatment, the tumors still remained (mean size of the tumor was $35.3 \text{ mm}^3$); these tumors were considered gefitinib-resistant tumors (GRTs) [12].

We previously revealed that the GRTs showed high expression of *OCT4* as well as the lung CSC marker *CD133* at both the mRNA and protein levels [12]. Moreover, neither the mutation of *EGFR* T790M nor amplification of the *MET* gene were found in PC9-GRTs as previously described [12]. As demonstrated in Figure 4A, PC9-GRTs displayed elevated *FBXW7* expression at mRNA level as determined by qPCR analysis. Immunohistochemical analysis showed upregulation of FBXW7 expression in the CD133-positive cells and downregulation of c-MYC expression in GRTs as compared with control tumors (Fig. 4B). We also examined the expression of FBXW7 in CD133-positive cells as well as c-MYC in pairs of pre-treated tumor specimens and specimens with acquired resistance to gefitinib from 14 NSCLC patients, which lacked the T790M mutation.

Immunohistochemical analysis demonstrated that the FBXW7 expression level in CD133-positive cells was higher and that of c-MYC was lower in nine of the 14 gefitinib resistant tumor specimens.
from NSCLC patients with EGFR mutation-positive as compared with the tumor specimens collected prior to treatment (Fig. 4C).

DISCUSSION

In this study, we developed gefitinib-resistant persisters (GRPs) by exposing PC9 and HCC827 cells to a high concentration of gefitinib harboring an activating EGFR mutation. GRPs expressed a high level of FBXW7 and CD133 as well as a low level of c-MYC in vitro. Cell cycle analysis revealed that the majority of GRPs existed in G0/G1 phase. GRPs expressed both FBXW7 and CD133 in the quiescent state, and knockdown of the FBXW7 gene in GRPs significantly decreased the CD133-positive cell population after gefitinib exposure and reversed the cell population in G0/G1 phase. We also demonstrated that gefitinib-resistant tumors (GRTs) of PC9 cells that remained after gefitinib treatment in mice also expressed a high level of FBXW7 in CD133-positive cells and a low level of c-MYC protein in vivo. FBXW7 expression was increased in CD133-positive cells while c-MYC expression was decreased in tumor specimens from acquired resistance of NSCLC patients with EGFR mutation-positive. These findings indicate that FBXW7 plays a crucial role in the maintenance of quiescence in GRPs in NSCLC with an activating EGFR mutation.

We previously showed that GRPs demonstrate high levels of expression of stem cell genes including CD133, OCT4, SOX2, NANOГ, CXCR4, and ALDH1A1, and they have distinctive profiles of CSC phenotype [11]. GRPs showed a high ability for formation of sphere in vitro and tumorigenicity in
In this study, we analyzed the cell cycle status by using FUCCI-expressing PC9 cells and FUCCI-expressing HCC827 cells. Cell cycle analysis revealed that the vast majority of GRPs from PC9-FUCCI and HCC827-FUCCI were in G0/G1 phase, while the majority of untreated cells were in S/G2/M phase. These findings strongly indicate that GRPs are very quiescent, exist in G0/G1 phase, and have characteristic features of the CSC phenotype.

The F-box protein FBXW7 is the substrate recognition subunit of the SCF-type ubiquitin ligase complex that is responsible for the degradation of many proteins, including c-MYC, via the ubiquitylation process [9, 17]. FBXW7 has been shown to play a role in maintaining quiescence by degradation of c-MYC protein. Silencing of FBXW7 in quiescent prostate cancer cells resulted in cell cycle re-entry via c-MYC protein accession [18]. Another study demonstrated that FBXW7 expression was upregulated in CSCs from colorectal cancer and that silencing of FBXW7 significantly increased the chemosensitivity of the colorectal CSCs and led to upregulation of c-MYC expression [19]. This evidence is consistent with our data that FBXW7 expression was upregulated in GRPs in NSCLC and that silencing of FBXW7 resulted in upregulation of the c-MYC expression level. We also found that FBXW7 expression was higher in CD133-positive cells and that c-MYC was lower in our in vivo GRT model after gefitinib treatment in mice. In addition, we demonstrated that FBXW7 expression in CD133-positive cells was increased and c-MYC expression was decreased in tumor specimens from NSCLC patients with acquired resistance to gefitinib as compared with tumor specimens collected prior to treatment. These findings are coherent with earlier
research which has demonstrated that a high FBXW7 expression level significantly correlates with low c-MYC expression in specimens of resected liver metastases from colorectal cancer after chemotherapy [19].

Leukemia-initiating cells (LICs) of chronic myeloid leukemia (CML) have been shown to be resistant to the tyrosine kinase inhibitor (TKI) imatinib, and quiescence in LICs of CML is thought to contribute to imatinib resistance [20]. FBXW7 is required for the maintenance of quiescence in LICs and that abrogation of quiescence in LICs by FBXW7 ablation sensitized LICs to imatinib [10]. In our study, knockdown of FBXW7 significantly reduced the number of CD133-positive GRPs and reversed the cell population in G0/G1-phase, indicating that FBXW7 plays a pivotal role in the maintenance and quiescence of GRPs.

Recently, it has been reported that silencing of FBXW7 induces gefitinib resistance in NSCLC cell lines through the regulation of the epithelial mesenchymal transition (EMT) and mTOR pathway [21]. Loss of FBXW7 leads to resistance to EGFR and ALK inhibitors via stabilization of MCL-1 in NSCLC cell lines [22]. However, these studies investigated a role of FBXW7 in the overall population of differentiated NSCLC cells. In our study, we focused on a small population of these cells with CSC properties, CD133-positive GRPs. Our present study suggests a different role for FBXW7 in the differentiated cells as compared with CSCs or tumor-initiating cells in gefitinib resistance of NSCLC with an activating EGFR mutation. However, further studies are necessary to clarify this finding.
Collectively, our study identifies a role for FBXW7 in the maintenance and quiescence of gefitinib-resistant persisters. Targeting FBXW7-mediated quiescence may represent a promising strategy to treat gefitinib-resistant lung CSCs in NSCLC with an EGFR mutation.

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DECLARATION OF INTERESTS

K.T. (Kazuhisa Takahashi) received research funding from Chugai Pharm, Ono Pharma, Taiho Pharm, Nippon Boehringer Ingelheim, AstraZeneca, Pfizer, MSD, and Lilly Japan. The remaining authors declare no conflict of interests.
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FIGURES

Hidayat M, et al. Figure 1
Figure 1. Gefitinib-resistant-persisters (GRPs) of PC9 and HCC827 cells express high levels of FBXW7 and CD133 but a low level of c-MYC. (A) The mRNA expression of FBXW7 and CD133 in PC9 and HCC827 cells was analyzed by quantitative polymerase chain reaction (qPCR) nine days after exposure to gefitinib. Expression levels of FBXW7 and CD133 in parental cells and in GRPs of PC9 and HCC827 were compared. Data were normalized to actin expression. All values represent the average of triplicate experiments. Average values for each parental cell line were set to 1. *p < 0.05, **p < 0.01. (B) Immunofluorescence of FBXW7 and c-MYC in PC9 and HCC827 cells. Cells were cultured with 1 μM gefitinib on slides of Labo-Tek chamber for 72 hours. Nuclei were stained with DAPI. (C) The ratio of FBXW7 positive or c-MYC positive cells in PC-9 and HCC827 cells. Parental cells and GRPs from each cell line were compared. Average values were calculated from five fields in each sample. **p < 0.01; scale bar indicates 50 μm.
Hidayat M, *et al.* Figure 2
Figure 2. Silencing of FBXW7 significantly decreased the population of CD133-positive cells and reversed c-MYC expression in PC9-GRPs. (A) FBXW7 expression was knocked down in PC9-GRPs with siRNA followed by gefitinib treatment for 72 hours and examined by qPCR. Two specific siRNAs and one non-specific control siRNA were used, and representative data from the siRNA experiment are shown. Average values for cells treated with the control siRNA were set to 1. Average values were calculated from triplicate experiments. Error bars, SD. **p < 0.01. (B, left) FBXW7 protein expression knocked down with siRNA in PC9-GRPs cells after gefitinib treatment for 72 hours was analyzed by immunofluorescence. Nuclei were stained with DAPI. (C, left) c-MYC expression was analyzed by immunofluorescence after silencing FBXW7 followed by gefitinib treatment for 72 hours in PC9-GRPs cells. DAPI (blue) was used to stain nuclei. (B, right and C, right) The ratio of FBXW7-positive cells (B, right) or c-MYC positive cells (C, right) in PC9-GRPs cells after silencing FBXW7. Average values were calculated from five fields in each sample. **p < 0.01, scale bar indicates 50 μm. (D) Knockdown of FBXW7 expression with siFBXW7 followed by gefitinib treatment for 72 hours in PC9-GRPs cells significantly suppressed CD133 expression as determined by q-PCR. Average values and SDs were calculated from triplicate samples. Average values for cells treated with the control siRNA were set to 1. **p < 0.01. (E, left) PC9 cells were cultured on slides of Labo-Tek chamber, and FBXW7 expression was silenced with siFBXW7. Cells were treated with 1 μM gefitinib for 72 hours. CD133-positive GRPs were significantly decreased. DAPI (blue) was used to stain nuclei. (E, right) The ratio of CD133-positive cells in PC9-GRPs
when silencing \textit{FBXW7} followed by gefitinib treatment for 72 hours. Average values were calculated from five fields in each sample $**p < 0.01$, scale bar indicates 50 μm.
Hidayat M, et al. Figure 3
**Figure 3.** The majority of GRPs of PC9-FUCCI exist in a quiescent state and maintained by FBXW7. Stable FUCCI-expressing cells were made by introducing FUCCI-vector plasmid into PC9. (A, left) PC9-FUCCI cells were seeded in 35 mm glass-bottom dishes, followed by treatment with or without gefitinib and subsequently subjected to cell cycle analysis. The cells in G0/G1 or S/G2/M phases appear red and green, respectively. (A, right) Histograms demonstrate the cell cycle phase distribution before and after gefitinib treatment. (B, left) The cell cycle profile of PC9-FUCCI was analyzed after knockdown of *FBXW7* using siRNA transfection and gefitinib treatment for 72 hours. (B, right) Histograms show the distribution of cells in G0/G1 phase and S/G2/M phase after knocking down FBXW7 under gefitinib treatment. Five pictures were taken from each group, and the cell numbers in G0/G1 phase as well as S/G2/M phase were counted and divided by the total number of cells. (C) The number of GRPs was decreased after knockdown of FBXW7 in PC9-FUCCI. Five pictures were taken from each group and the number of cells in G0/G1 phase as well as in S/G2/M phase were counted. Scale bar indicates 50 μm.
Figure 4. The expression of FBXW7 in CD133-positive cells was higher and that of c-MYC was lower in gefitinib-resistant tumors (GRT) of PC9 in vivo as well as in lung cancer specimens from NSCLC patients who had acquired resistance to gefitinib. (A) PC9 cells were injected into NOG mice subcutaneously followed by intraperitoneal injection (six times/week) of 20 mg/kg of gefitinib or vehicle after the tumor volume reached 75 mm$^3$. After 14 to 17 days of gefitinib treatment, the tumors still remained (mean size of the tumor was 35.3 mm$^3$). Tumors were taken, and qPCR was carried out with FBXW7 primer on PC9 tumors and PC9-GRT. Data were normalized to actin expression and represent mean ± SD. **$p < 0.01$. (B, left) The expression levels of FBXW7 in CD133-positive cells and c-MYC in PC9 tumors and PC9-GRTs were examined by immunohistochemistry. (B, right) The ratio of FBXW7 and CD133-positive cells and c-MYC-positive cells. Cell nuclei were stained with DAPI (blue). Images were obtained using a ZEISS LSM 780 system. Five pictures were taken from each group and the number of cells expressing FBXW7 and c-MYC in CD133-positive cells were counted *$p < 0.05$, **$p < 0.01$. Scale bar indicates 25 μm. (C, top) The expression of FBXW7 in CD133-positive cells and c-MYC in pairs of pre-treated tumor specimens and specimens with acquired resistance to gefitinib from NSCLC patients were examined by immunohistochemistry. (C, bottom) The ratio of FBXW7 in CD133-positive cells and c-MYC-positive cells. Cell nuclei were stained with DAPI (blue). Images were obtained using a ZEISS LSM 780 system. Five pictures were taken from each group, and the number of CD133-positive cells that expressed FBXW7 and c-MYC were counted. *$p < 0.05$, **$p < 0.01$. Scale bar indicates 25 μm.
Figure S1. Silencing of *FBXW7* significantly decreased the population of CD133-positive cells and...
reversed c-MYC expression in HCC827-GRPs. (A) FBXW7 expression was knocked down in HCC827-GRPs with siRNA followed by gefitinib treatment for 72 hours and examined by qPCR. Two specific siRNAs and one non-specific control were used, and data representative of the siRNA experiment are shown. Average values for cells treated with the control siRNA were set to 1. Average values were calculated from triplicate experiments. Error bars, SD. **p < 0.01. (B, left) FBXW7 expression at protein level was knocked down with siRNA in HCC827-GRPs and analyzed by immunofluorescence after gefitinib treatment for 72 hours. Nuclei were stained with DAPI. (C, left) c-MYC expression was analyzed by immunofluorescence after silencing of FBXW7 and gefitinib treatment for 72 hours in HCC827-GRPs. Nuclei were stained with DAPI. (B, right and C, right) The ratio of FBXW7 positive cells (B, right) or c-MYC positive cells (C, right) in HCC827-GRPs cells following silencing of FBXW7. Average values were calculated from five fields in each sample. **p < 0.01, scale bar indicates 50 μm. (D) Knockdown of FBXW7 expression with siFBXW7 followed by gefitinib treatment for 72 hours in HCC827-GRPs significantly suppressed CD133 expression as determined by q-PCR. Average values and SDs were calculated from triplicate samples. Average values for cells treated with the control siRNA were set to 1. **p < 0.01. (E, left) HCC827 cells were cultured on Labo-Tek chamber slides, and FBXW7 expression was silenced with siFBXW7 after which cells were treated with 1 μM gefitinib for 72 hours. The number of CD133-positive GRPs was significantly decreased. Nuclei were stained with DAPI (blue). (E, right) The ratio of CD133 positive cells in HCC827-GRPs following FBXW7 followed by gefitinib treatment for 72 hours silencing.
Average values were calculated from five fields in each sample. **p < 0.01, scale bar indicates 50 μm.

**Figure S2.** Both FBXW7 and CD133 proteins were highly expressed in GRPs of PC9-FUCCI and HCC827-FUCCI. The fluorescence image of GRPs showed red color, indicating that GRPs existed in G0/G1 phase. Cells were seeded in 35 mm glass-bottom dishes, followed by treatment with or without 1 μM gefitinib for 72 hours. Scale bar indicates 20 μm.
**Figure S3.** The majority of GRPs of HCC827-FUCCI existed in a quiescent state and were maintained by FBXW7. Stable FUCCI-expressing cells were made by introducing FUCCI-vector plasmid into HCC827. (A, left) HCC827-FUCCI cells were seeded in 35 mm glass-bottom dishes,
followed by treatment with or without gefitinib and subsequently were subjected to cell cycle analysis. The cells in G0/G1 or S/G2/M phases appear red and green, respectively. (A, right) Histograms demonstrate the distribution of the cell cycle phases before and after treatment with gefitinib. (B, left) The cell cycle profile of HCC827-FUCCI was analyzed following knockdown of FBXW7 by using reverse-siRNA transfection and gefitinib treatment for 72 hours. (B, right) Histograms show the distribution of G0/G1 phase and S/G2/M phase after knockdown of FBXW7 under gefitinib treatment. Five images were taken from each group, and the cell population in G0/G1 phase as well as S/G2/M phase was counted and divided by the total number of cells. (C) The number of GRPs was decreased following knockdown of FBXW7 in HCC827-FUCCI. Five images were taken from each group and the number of cells in G0/G1 phase as well as S/G2/M phase were counted. Scale bar indicates 50 μm.

**Supplemental materials and methods**

**Cell culture and reagents**

Two NSCLC cell lines, PC9 and HCC827, which express EGFR exon 19 deletion mutations (ΔE746-A750), were used in this study. PC9 cells were established at the Tokyo Medical University (Tokyo, Japan) as previously described [1] and were kindly provided by Dr. Kazuto Nishio (Department of Genome Biology, School of Medicine; Kinki University, Osaka). HCC827 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Cell lines were verified to be
mycoplasma free. Cells were cultured in RPMI-1640 medium (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin (100 U/mL and 100 µg/mL, respectively), and were grown in a humidified 5% CO$_2$ atmosphere at 37°C in an incubator, in which the oxygen tension was held at 21% (normoxia). Gefitinib was purchased from JS Research Chemicals Trading (Wedel, Germany).

**Quantitative real-time PCR**

Total RNA was extracted from cell lines using the mirVana miRNA Isolation kit (Ambion, Austin, TX, USA). cDNA was created from 1 µg of RNA using the Revertra cDNA synthesis kit (Toyobo, Osaka, Japan) according to the manufacturer’s protocol. Quantitative real-time PCR (qPCR) was carried out using the Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) as previously described [2]. The following program was used: holding at 95°C for 20 sec and amplification via 40 cycles (denaturation 95°C for 3 sec, annealing, and extension at 60°C for 30 sec), with concurrent melt-curve analysis. Actin served as an internal control.

The following primers were used:

**CD133**

Forward, 5′-GGCCCAGTACAAACACTACCAA-3′

Reverse, 5′-CGCCTCCTAGCACTGAATTGATA-3′

**FBXW7**
Forward, 5′-CGACGCGAATTACATCTGTC-3′
Reverse, 5′-CGTTGAAACTGGGGTTCTATCA-3′

Actin
Forward, 5′-CTCTTCCAGCTTCCTTCT-3′
Reverse, 5′-AGCAGCTGTGTTGCGTACAG-3′

**RNA interference**

Small interfering RNAs (siRNAs) targeting FBXW7 (Stealth Select RNAi siRNA) were custom synthesized by Invitrogen. A negative control was also purchased from Invitrogen. To exclude off-target effects, PC9 cells and HCC827 cells were transfected with two different specific siRNAs and one non-specific control using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instructions. The cells were detached and diluted in complete growth medium without antibiotics and then plated in each of the wells. RNAi duplex and lipofectamine RNAiMAX were mixed in Opti-MEM®I (Gibco) reduced serum medium and incubated for 15 minutes at room temperature. RNAi duplex-Lipofectamine™ RNAiMAX complexes were added to the wells containing cells. The cells were then incubated for 24 h at 37°C followed by gefitinib treatment for 72 hours. The sequences of the siRNA against FBXW7 were as follows:

Stealth FBXW7 oligo #1: 5′-GAGACUUCAUUUCAUGCUCCUA-3′;
Stealth FBXW7 oligo #2: 5′-AGUUGGCACUCUAUGUGCUUCAUU-3′
**Immunofluorescence**

Cells were cultured with 1 μM gefitinib for 72 h, fixed with 4% paraformaldehyde for 5 min, and permeabilized with 0.1% Triton X-100 for 5 min. After blocking with 10% goat serum in phosphate-buffered saline (PBS) for 1 hour at room temperature, cells were incubated at 4°C overnight with the primary antibody: FBXW7 (Abcam, [Cambridge, United Kingdom]), c-MYC (Cell Signalling Technology, [Danvers, Massachusetts, USA]), and CD133 (Milenyi Biotec, Bergisch Gladbach, Germany). Protein was visualized by incubation with secondary antibody labeled with Alexa Fluor 594 goat anti-mouse IgG, Alexa Fluor 488 goat anti-mouse IgG, and Alexa Fluor 647 goat anti-mouse and anti-rabbit IgG (Invitrogen). Slides were mounted using Vectashield Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA). The images were obtained with a confocal microscope (ZEISS LSM 780) outfitted with 488 nm (argon), 561 nm (red DPSS), 633 (He/Ne) using 40X water objective and with ZEN 2 black edition software (ZEISS). Alternatively, an Axioplan 2 imaging system was used (ZEISS, Oberkochen, Germany) with AxioVision software (ZEISS). Images used for comparisons of different cells and/or treatments were acquired with the same instrument settings and exposure times and were processed similarly. The number of FBXW7, c-MYC, and CD133-positive cells was counted; the ratio of positive cells was calculated in five fields for each experiment. As for transient knockdown of FBXW7, PC9 and HCC827 were grown on Lab-Tek chamber II slides (Nunc, Rochester, NY, USA) and transiently knocked down by using
reverse-siRNA transfection and treated with 1 μM gefitinib for 72 h, and the rest of the methods were the same. For the double staining of FBXW7/CD133 in PC9-FUCCI and HCC827 FUCCI, cells were grown on 35 mm glass-bottom dishes (Greiner Bio-One), permeabilized with 0.2% tween 20 for 20 minutes, and FBXW7 protein was visualized by incubation with secondary antibody labeled with Alexa Fluor 450 goat anti-mouse IgG (Invitrogen). DAPI staining was not used in this experiment. The rest of the methods were the same.

**FUCCI (fluorescence ubiquitination cell cycle indicator)**

pFucci-S/G2/M green and pFucci-G1 orange plasmid were purchased from MBL (Nagoya, Japan). *Fucci-S/G2/M green (mKO2-hCdt1) and Fucci-G1 orange (mAG-hGem)* were amplified by polymerase chain reaction (PCR) using LA Taq DNA Polymerase (TaKaRa Bio, Kyoto, Japan), and they were linked in-frame linked a T2A sequence [3]. Then, the *Fucci-S/G2/M green-T2A-Fucci-G1 orange* fusion gene was cloned into the lentiviral vector CSII-CMV (kindly provided by Dr. Miyoshi, RIKEN BioResource Center, Tsukuba, Japan), and the resulting plasmid was designated as CSII-CMV-FUCCI-S/G2/M green-G1 orange. The expressing plasmid of CSII-CMV-FUCCI-S/G2/M green-G1 orange was mixed with packaging plasmids and transfected into 293T cells (Invitrogen). The supernatant medium was collected 2 days after transduction and filtered through a 0.45µm membrane (Millipore). Then PC9 and HCC827 cells were infected using 6 µg/mL of polybrene as previously described [4]. The successfully transfected cells were identified by using confocal
microscope (ZEIS LSM 780). FUCCI-expressing positive cells were used for further experiments.

**Mice**

Seven-week-old female NOD/Shi-scid/IL-2Rcnull (NOG) mice were obtained from the Central Institute for Experimental Animals (Kanagawa, Japan). All mice were shipped to Juntendo University and were maintained under pathogen-free conditions. The mice were housed in a room under controlled temperature (25°C), humidity, and lighting (12-h light/dark cycle). *Ad libitum* access to food and tap water was allowed throughout the study period.

**Establishment of gefitinib-resistant tumors (GRTs) in vivo**

We implanted $1 \times 10^5$ PC9 cells into NOG mice, and once tumors reached the size of $75 \text{ mm}^2$, we began treatment of these mice with gefitinib (20mg/kg) by intraperitoneal injection (6 times/week). After 14 days of gefitinib treatment, the tumors remained, and these tumors were referred to as gefitinib-resistant tumors (GRTs). Tumors were collected 14 days after gefitinib treatment and were subjected to *FBXW7* expression analysis by qPCR and expression of *FBXW7* and c-MYC by immunohistochemistry.

**Immunohistochemistry**

Paraffin-fixed GRTs samples and lung cancer specimens were subject to the following steps:
deparaffinization, antigen retrieval, blocking, and immunohistochemical staining with FBXW7 antibody at a 1:1000 dilution (Abcam, Cambridge, United Kingdom) and with c-MYC antibody at a 1:200 dilution (Abcam). Slides were incubated at 4°C overnight with primary antibody. Protein was visualized by incubation with secondary antibody labeled with Alexa Fluor 594 goat anti-mouse IgG (Invitrogen) and Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen). Slides were mounted using Vectashield Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA). The number of both FBXW7 and CD133 as well as c-MYC-positive cells were counted; the ratio of positive cells was calculated in five fields for each experiment. Images were obtained by using a confocal microscope (ZEIS LSM 780) using 40X water objective and with ZEN 2 black edition software (ZEISS).

**Ethics**

All animal experiments were carried out in accordance with the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology of Japan (Notice No. 71, 2006) and were approved by the Committee for Animal Experimentation of Juntendo University with the Approval No. 240182. Tumor specimens were obtained from NSCLC patients before gefitinib treatment and after acquisition of resistance at Juntendo University Hospital with patients’ consent under an Institutional Review Board-approved protocol.
Statistical analysis

Statistical analyses were performed in GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA).

Values were compared using a two-tailed Student’s *t*-test and ANOVA. Differences between the means were considered statistically significant when *p* < 0.05.

References


