FBXW7 Mediates Chemotherapeutic Sensitivity and Prognosis in NSCLCs
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Abstract
Non–small cell lung cancer (NSCLC) is a leading cause of cancer-related deaths worldwide. To improve the prognosis of patients with NSCLCs, new and validated therapeutic targets are critically needed. In this study, we focused on F-box and WD repeat domain containing-7 (FBXW7), an E3 ubiquitin ligase, that regulates the degradation of MCL1, Myc, cyclin E, and TOP2A. Importantly, loss of FBXW7 was associated with increased sensitivity of tumors to a class I–specific histone deacetylase (HDAC) inhibitor, MS-275. Immunohistochemical analysis revealed increased expression of FBXW7 targets, MCL1 and TOP2A, in NSCLC tumors with low expression of FBXW7. Moreover, clinical specimens exhibiting low FBXW7 expression presented with more progressive cancer and significantly shorter cancer-specific survival than patients with high FBXW7 expression. Mechanistic study of NSCLC cell lines with silenced FBXW7 revealed enhanced MS-275 sensitivity and taxol resistance. Interestingly, taxol resistance was eliminated by MS-275 treatment, suggesting the potential of HDAC inhibitors for the treatment of aggressive taxol-resistant NSCLCs that lack FBXW7.

Implications: FBXW7 status impacts chemosensitivity and is a prognostic marker in NSCLCs.

Visual Overview: http://mcr.aacrjournals.org/content/early/2013/12/19/1541-7786.MCR-13-0341. Mol Cancer Res; 1–6. © 2013 AACR.

Introduction
Lung cancer is a leading cause of cancer-related deaths worldwide, and non–small cell lung cancer (NSCLC) represents approximately 85% of all cases of lung cancer (1). Recently, targeted therapies based on EGF receptor (EGFR)-activating mutations and EML4-ALK gene rearrangements have become standard therapeutic tools, and this approach has resulted in the extension of survival rates for patients with NSCLCs (2). However, patients with advanced NSCLCs who would not respond to a targeted therapy have poor prognoses. To improve the prognosis in patients with NSCLCs, further research is required worldwide to identify new therapeutic targets.

In this study, we focused on the F-box and WD-40 domain protein 7 gene (FBXW7), which encodes a tumor suppressor that forms 1 of the 4 subunits of SCF ubiquitin ligase complexes and induces the degradation of oncoproteins such as MCL1, Myc, cyclin E, mTOR, and TOP2A (3–7). Clinically, low expression of FBXW7 in human solid tumors such as colorectal cancer and gastric cancer is related to cancer progression and poor prognosis because of the accumulation of the abovementioned oncoproteins (8). In lung cancer, mutations in FBXW7 and a low copy number of FBXW7 have been reported to be associated with low levels of FBXW7 expression and with resistance to antitubulin chemotherapeutics via accumulation of MCL1 (5). These findings indicate that FBXW7 plays very important roles in cancer progression and refractory disease in NSCLCs. However, few studies have addressed the clinical significance of FBXW7 in patients with NSCLCs.

Recently, Garnett and colleagues determined the correlations between gene mutations and sensitivity to 130 drugs under clinical and preclinical investigation by testing a panel of several hundred cancer cell lines. This study revealed that cancer cell lines deficient for FBXW7 are highly sensitive to the histone deacetylase (HDAC) inhibitor MS-275 (9). Moreover, therapy with the HDAC inhibitor vorinostat and the BCL2 inhibitor ABT-737 had a synergistic anticancer effect in squamous cell carcinoma cell lines with mutations in FBXW7 (10). These data suggest that in several cancers, including NSCLCs, aggressive tumors that display loss of FBXW7 might be controlled by treatment with HDAC inhibitors such as MS-275.
The purpose of this study was to clarify the clinical significance of the tumor suppressor FBXW7 in NSCLCs and to examine the usefulness of MS-275 treatment in NSCLC cell lines. Therefore, immunohistochemistry was conducted in clinical NSCLC samples to evaluate the relationship between FBXW7 expression and clinicopathologic features. Moreover, we conducted an RNA interference (RNAi) analysis of FBXW7 to determine whether this gene plays a role in the sensitivity of NSCLC cells to MS-275 and anti-tubulin chemotherapeutics.

Materials and Methods
Clinical samples and cell lines
We analyzed tumor specimens from 103 patients with lung cancer who underwent surgery for excision of a primary tumor between March 1999 and September 2005 in the department of General Surgical Science of Gunma University School of Medicine (Maebashi, Japan). The patients included 69 men and 34 women with a median age at surgery of 65 years (range, 32–84 years). Sixty-seven patients were former/current smokers, with a median Brinkman index (BI; number of cigarettes per day times years) of 673; 34 of the remaining 36 patients had never smoked and the smoking status of 2 was unknown. Seventy-three patients had adenocarcinomas, 18 had squamous cell carcinomas, and 9 had other types of carcinoma. Sixty-one patients had stage I, 9 had stage II, 29 had stage III, and 4 had stage IV lung cancer at the time of surgery.

The human lung cancer cell lines H2009 and H1975 were maintained in RPMI-1640 medium containing 10% FBS and 100 units/mL penicillin and streptomycin sulfates and cultured in a humidified incubator with 5% CO2 at 37°C. These cell lines were obtained from the American Type Culture Collection.

Immunohistochemistry
The resected surgical specimens were fixed with 10% formaldehyde, embedded in paraffin blocks, cut into 4-μm-thick sections, and mounted on glass slides. The staining procedure was conducted by using standard methods described previously (11). The sections were then incubated overnight at 4°C and at room temperature for 30 minutes with mouse monoclonal anti-FBXW7 antibody (Abnova) at a dilution of 1:200 in PBS containing 1% bovine serum albumin. The sections were lightly counterstained in Mayer’s hematoxylin and mounted on glass slides.

The level of FBXW7 immunoreactivity was defined as follows: (i) low expression = no staining or weak-to-strong complete nuclear staining in <20% of tumor cells and (ii) high expression = weak-to-strong complete nuclear staining in ≥20% of tumor cells.

Mutation analysis
After obtaining appropriate approval from the institutional review board and written informed consent from the patients, the tumor specimens were rapidly frozen in liquid nitrogen after resection and stored at −80°C. Total RNA was isolated by using the RNeasy Kit (Qiagen). After cDNA was synthesized, we searched for mutations in exons 18 through 21, which encode the tyrosine kinase domains of the EGFR and KRAS genes, as described previously (12, 13).

RNAi of FBXW7 expression
FBXW7-specific siRNA (sense sequence: GCACAGAA-UUGAUACUAACCT; antisense sequence: GUUAGUA-UCAAUCUGUGCTG) and a negative control siRNA (siTrio Negative Control 1; scrambled siRNA) were purchased from Bonac and Hokkaido System Science, respectively. The H2009 and H1975 lung cancer cell lines were seeded at 2 × 104 cells per well in a volume of 2 mL in 6-well flat-bottom plates and then incubated in a humidified atmosphere (37°C and 5% CO2). After 24 hours of incubation, 200 μL of Opti-MEM I Reduced-Serum Medium (Life Technologies Corporation), 4 μL of Lipofectamine RNAi MAX (Life Technologies Corporation), and the siRNA (scrambled siRNA or FBXW7 siRNAs) were mixed and incubated for 10 to 20 minutes. Then, the reagents and 800 μL of Opti-MEM I were added to each well. RNAi assays were conducted after a 72-hour incubation period.

Drug sensitivity assay
An analysis of drug sensitivity was conducted by using cells that had been transfected with siRNA targeting the mRNA of the FBXW7 gene. The cells were plated in 96-well plates at approximately 5,000 cells per well with 100 μL of medium. To quantitate cell viability with the water-soluble tetrazolium (WST)-8 assay (Dojindo Laboratories), 10 μL of the cell-counting solution was added to each well after 48 hours and the plates were incubated at 37°C for 2 hours. The cell proliferation rate was determined by measuring the absorbance of the medium at 450 nm with the reference wavelength set at 650 nm. The absorbances were read by using a microtiter plate reader (Molecular Devices). Taxol and MS-275 were purchased from Bristol-Myers Squibb and Selleck Chemicals, respectively.

Protein extraction and Western blot analysis
Western blot analysis was used to confirm the expression of FBXW7 (Abnova), TOP2A (Abcam), MCL1 (Origene), and β-actin in the lung cancer cell lines. Total proteins were extracted with PROPREP protein extraction solution (iNtRON Biotechnology, Inc.). Total protein was electrophoresed through NuPAGE 4%–12% Bis–Tris gels (Life Technologies Corporation) and then electrotransferred to polyvinylidene difluoride (PVDF) membranes by using an iBlot Gel Transfer Device. The membranes were blocked with 5% skim milk and the proteins were detected by using an anti-FBXW7 mouse monoclonal antibody (1:1,000), an anti-MCL1 rabbit monoclonal antibody (1:1,000), and an anti-TOP2A rabbit monoclonal antibody (1:1,000); and an anti-β-actin mouse monoclonal antibody (clone AC-74; Sigma; 1:1,000) served as a control. Bands were detected, and band intensities were calculated by using ECL Prime.
Western Blotting Detection Reagent and an Image Quant LAS 4000 (GE Healthcare Life Sciences).

Statistical analysis
Statistical analyses were conducted by using the $t$ test for continuous variables and the $\chi^2$ test for categorical variables. Survival curves were generated according to the Kaplan–Meier method. The differences between survival curves were examined by using the log-rank test. In addition, univariate and multivariate survival analyses were conducted by using the Cox proportional hazards model. ANOVA was used to assess the statistical significance of the results of the in vitro assays. A result was considered statistically significant when relevant $P < 0.05$. All statistical analyses were conducted with JMP 5.0 software (SAS Institute Inc.).

Results
Immunohistochemical analysis of the expression of FBXW7 in NSCLC tissues
We used immunohistochemistry to investigate the nuclear expression of FBXW7 in 103 NSCLC specimens. Seventy-two (69.9%) NSCLC specimens were assigned to the high-FBXW7-expression group and 31 (30.1%) were assigned to the low-FBXW7-expression group. Representative results of the immunohistochemical experiments are shown in Fig. 1. Our analysis revealed enhanced expression of MCL1 and TOP2A in specimens of the low-FBXW7-expression group (Fig. 1A). Conversely, MCL1 and TOP2A expression levels were decreased in specimens of the high-FBXW7-expression group (Fig. 1B). The relationship between FBXW7 expression and the target proteins in the clinical NSCLC tissues was validated. In normal lung, immunostaining of FBXW7 was detected in nuclei, similar to that of FBXW7 expression in tumor cells (data not shown).

Association between FBXW7 expression and clinicopathologic features of NSCLCs
The correlations between FBXW7 expression in the NSCLC specimens and 10 clinicopathologic characteristics of the patients (gender, age, smoking status, Brinkman index, pathology, T factor, N factor, EGFR mutation status, KRAS mutation status, and clinical stage) are shown in Supplementary Table S1. One clinicopathologic factor was significantly different between the 2 groups: Patients with tumors that were assigned to the low-FBXW7-expression group ($n = 34$) had a more progressive T factor than those with tumors that were assigned to the high-FBXW7-expression group ($n = 69; P < 0.05$). However, no significant differences were observed regarding gender, age, smoking status, Brinkman index, pathology, N factor, EGFR mutation status, KRAS mutation status, or clinical stage.

Prognostic significance of FBXW7 expression in patients with NSCLCs
The cancer-specific survival rates of patients with tumors that were assigned to the low-FBXW7-expression group were significantly lower than those of patients with tumors that were assigned to the high-FBXW7-expression group ($P < 0.001$; Fig. 1C). In multivariate analysis, the low
expression of FBXW7 in NSCLC tissues was an independent prognostic factor for poor survival, as was the T factor ($P = 0.031$; Supplementary Table S2).

RNAi of FBXW7 enhances MS-275 sensitivity and taxol resistance in vitro

Because downregulation of FBXW7 expression in cancer tissues is associated with poor prognosis, we used an siRNA to silence FBXW7 in the NSCLC cell lines H2009 and H1975. FBXW7 silencing was confirmed by Western blot analyses of the cells treated with control siRNA (the control-siRNA group) or with the FBXW7 siRNA (the FBXW7-siRNA group; Fig. 2B and D). Evaluation of the effects of MS-275 treatment revealed that the viability of the FBXW7-siRNA group was significantly decreased compared with that of the control-siRNA group and the cells that were not treated with any siRNA (Fig. 2A and C; left column). Moreover, after taxol treatment, the viability of the FBXW7-siRNA group was significantly enhanced compared with that of the control-siRNA group (Fig. 2A and C; right column).

Western blot analysis was used to determine the expression of MCL1 and cyclin E, both of which are degradation targets of FBXW7, in H1975 and H2009 cells treated with FBXW7 siRNA. The expression levels of both proteins were enhanced in the FBXW7-siRNA group compared with those of the control-siRNA group (Fig. 2B and D).

Treatment with MS-275 in vitro eliminates taxol resistance in cells with silenced FBXW7 expression

Evaluation of viability in the FBXW7-siRNA group after simultaneous treatment with taxol (0, 50, 250, and 1,000 μmol/L) and MS-275 (5 μmol/L) revealed that the taxol resistance observed in H1975 cells with silenced FBXW7 expression was eliminated by treatment with MS-275 (Fig. 3).

Discussion

In this study, we determined that low levels of FBXW7 expression are associated with cancer progression and that FBXW7 expression is an independent prognostic factor in patients with NSCLCs. These results are consistent with...
those of previous studies of several other types of cancer. Moreover, we showed that FBXW7 expression regulates the in vitro sensitivity of NSCLC cell lines to MS-275 and antitubulin chemotherapeutics.

**FBXW7**-deficient mice die in utero of vascular abnormalities at embryonic days 10.5 to 11.5 (14). However, mice with mutations of **FBXW7** at specific arginine residues that are involved in substrate binding develop normally in utero but die perinatally because of a defect in lung development that is caused by accumulation of Tgif1 and Klf5 (15). Moreover, although *p53*-deficient mice do not develop epithelial tumors, loss of **FBXW7** alters the tumor profile in these mice and they develop lung cancer (16). In addition, suppression and mutation of the tumor suppressor **FBXW7** have been reported in several types of epithelial malignancies (8, 11, 17). Previous reports have suggested that **FBXW7** might be important in lung development and lung carcinogenesis.

**FBXW7** functions as a tumor suppressor by controlling the degradation of oncoproteins such as MYC, cyclin E, TOP2A, and MCL1, which are all associated with tumor progression and chemoresistance. In our clinical NSCLC samples, low expression of **FBXW7** was associated with cancer progression (as indicated by the T factor) and poor prognosis; however, expression was not associated with other existing clinicopathologic factors or with mutations in EGFR or KRAS. In the clinical NSCLC tissues that we characterized, the expression of TOP2A and MCL1 was elevated in cells with low levels of **FBXW7** expression. Suppression of **FBXW7** expression in NSCLCs is thought to be associated with cancer progression and to promote resistance of chemotherapeutics, including antitubulin therapies via accumulation of MCL1 and several other oncoproteins (5). In this study, we validated the finding that cells with low levels of **FBXW7** expression are related to the proliferation potency (Supplementary Fig. S1) and resistant to taxol. Mutations in **FBXW7** are reported to be rare in lung adenocarcinoma (18); however, **FBXW7** expression in solid cancers is regulated by copy number variation, p53 transcriptional activity, and microRNAs (8, 11, 19, 20). Cancer cells that express low levels of **FBXW7** in the absence of **FBXW7** mutations might be killed by HDAC inhibitors via the upregulation of transcriptional activity for **FBXW7**, p53, or microRNAs. Cell lines with low or suppressed expression of **FBXW7** have been reported to be highly sensitive to the HDAC inhibitor MS-275 (9). MS-275 treatment has been reported to reduce DNA double-strand break repair and cell growth (21, 22). Moreover, treatment with MS-275 suppressed the expression of **MCL1** that is associated with taxol resistance and induced the expression of **FBXW7** and p21 (Supplementary Fig. S2; ref. 23). In this study, we administered MS-275 to NSCLC cells with silenced **FBXW7** to focus on the recovery of sensitivity to the antitubulin drug taxol (Fig. 3). MS-275 and the induced **FBXW7** might work cooperatively to suppress MCL1. In the clinic, resistance of NSCLC tumors to antitubulin chemotherapy is a very important problem that must be overcome. The therapeutic approach of combining conventional chemotherapy with the HDAC inhibitor MS-275 might be useful for patients with a poor prognosis whose NSCLC shows suppressed **FBXW7** expression.

In conclusion, we have discovered that a low level of **FBXW7** expression could be a powerful marker of poor prognosis in patients with NSCLCs and that the sensitivity of tumor cells to the HDAC inhibitor MS-275 and antitubulin chemotherapeutics is regulated by **FBXW7**. MS-275 may be a promising therapeutic tool to overcome the resistance of NSCLCs to taxol chemotherapy.

**Disclosure of Potential Conflicts of Interest** No potential conflicts of interest were disclosed.

**Authors’ Contributions**
Conception and design: T. Yokobori, Y. Yokoyama, T. Miyazaki, H. Kuwano Development of methodology: T. Yokobori, Y. Yokoyama, H. Kuwano Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T. Yokobori, Y. Yokoyama, B. Alttan, T. Kosaka, E. Yamaki, S. Tanaka, H. Kuwano, R. Onozato, H. Endoh Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T. Yokobori, Y. Yokoyama, H. Kuwano Writing, review, and/or revision of the manuscript: T. Yokobori, A. Mogi, T. Miyazaki Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Yokoyama, T. Yajima, K. Tomizawa, Y. Åzuma, R. Onozato Study supervision: A. Mogi, H. Kuwano

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