FBXW7 Mediates Chemotherapeutic Sensitivity and Prognosis in NSCLCs

Takehiko Yokobori, Yozo Yokoyama, Akira Mogi, Hideki Endoh, Bolag Altan, Takayuki Kosaka, Ei Yamaki, Toshihiko Yajima, Kenji Tomizawa, Yoko Azuma, Ryoichi Onozato, Tatsuya Miyazaki, Shigebumi Tanaka, and Hiroyuki Kuwano

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/F1.large.jpg, Mol Cancer Res; 12(1); 32–37. © 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 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.

Authors’ Affiliation: Departments of General Surgical Science, Graduate School of Medicine, Gunma University, Showamachi, Maebashi, Japan

Note: Supplementary data for this article are available at Molecular Cancer Research Online (http://mcr.aacrjournals.org/).

Corresponding Author: Takehiko Yokobori, Department of General Surgical Science, Graduate School of Medicine, Gunma University, 3-39-22 Showamachi, Maebashi 371-8511 Japan. Phone: 81-027-220-8224; Fax: 81-027-220-8230; E-mail: bori45@gunma-u.ac.jp

doi: 10.1158/1541-7786.MCR-13-0341
©2013 American Association for Cancer Research.
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 antitubulin 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. 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 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: GCACAGAAGUGAUACUAACCTT; antisense sequence: GGUAGUAGUCUUUCUGUGCTG) 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 × 10^5 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 RNAiMAX (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

![Figure 1](https://example.com/image1.png)

**Figure 1.** Immunohistochemical analysis of FBXW7, MCL1, and TOP2A expression in representative NSCLC tissue samples. A, MCL1 (middle column) and TOP2A (right column) expression levels were enhanced in tissues that displayed low levels of FBXW7 expression (left column). B, MCL1 (middle column) and TOP2A (right column) expression levels were decreased in tissues that displayed high levels of FBXW7 expression (left column; 100× magnification; scale bar, 200 µm). C, Kaplan–Meier survival curves of patients with NSCLCs according to FBXW7 expression. The survival rate of patients with tumors that were assigned to the low-FBXW7-expression group ($n = 34$) was significantly lower than that of patients with tumors that were assigned to the high-FBXW7-expression group ($n = 69; P < 0.001$).
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 H2009 (B) and H1975 (D) cells treated with an siRNA targeting FBXW7 mRNA. $\beta$-Actin was used as a loading control. Band intensities were measured with an Image Quant LAS 4000. n.c., negative control ($*, P < 0.05$).

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 $\mu$mol/L) and MS-275 (5 $\mu$mol/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
Moreover, although that is caused by accumulation of Tgif1 and Klf5 (15).

Nakayama KI, Nakayama K. Ubiquitin ligases: cell-cycle control and


References


Mol Cancer Res; 12(1) January 2014 Molecular Cancer Research

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

Authors’ Contributions
Concept and design: Y. Yokoyama, 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. Altan, T. Kosaka, E. Yamaki, S. Tanaka, H. Kuwano, R. Oono, 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): T. Yokobori, Y. Tajima, K. Tomita, Y. Azuma, R. Oono
Study supervision: A. Mogi, H. Kuwano

Acknowledgments
The authors thank Yukie Saito, Tomoko Yano, Yuka Matsui, and Ayaka Ishida for their excellent assistance.

Grant Support
This work was supported, in part, by the following grants and foundations: Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS); grant numbers 2519690, 22591450, 23591857, and 30546726.

Received June 28, 2013; revised October 1, 2013; accepted October 9, 2013; published OnlineFirst October 28, 2013.


FBXW7 Mediates Chemotherapeutic Sensitivity and Prognosis in NSCLCs

Takehiko Yokobori, Yozo Yokoyama, Akira Mogi, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1541-7786.MCR-13-0341

Supplementary Material
Access the most recent supplemental material at:
http://mcr.aacrjournals.org/content/suppl/2013/10/28/1541-7786.MCR-13-0341.DC1

Visual Overview
A diagrammatic summary of the major findings and biological implications:
http://mcr.aacrjournals.org/content/12/1/32/F1.large.jpg

Cited articles
This article cites 23 articles, 8 of which you can access for free at:
http://mcr.aacrjournals.org/content/12/1/32.full#ref-list-1

Citing articles
This article has been cited by 6 HighWire-hosted articles. Access the articles at:
http://mcr.aacrjournals.org/content/12/1/32.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://mcr.aacrjournals.org/content/12/1/32.
Click on “Request Permissions” which will take you to the Copyright Clearance Center's (CCC) Rightslink site.