RESEARCH ARTICLE

FBW7 Upregulation Enhances Cisplatin Cytotoxicity in Nonsmall Cell Lung Cancer Cells

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Abstract

Introduction: Lung cancer is extremely harmful to human health and has one of the highest worldwide incidences of all malignant tumors. Approximately 80% of lung cancers are classified as non-small cell lung cancers (NSCLCs). Cisplatin-based multidrug chemotherapy regimen is standard for such lesions, but drug resistance is an increasing problem. F-box/WD repeat-containing protein 7 (FBW7) is a member of the F-box protein family that regulates cell cycle progression, and cell growth and differentiation. FBW7 also functions as a tumor suppressor. Methods: We used cell viability assays, Western blotting, and immunofluorescence combined with siRNA interference or plasmid transfection to investigate the underlying mechanism of cisplatin resistance in NSCLC cells. Results: We found that FBW7 upregulation significantly increased cisplatin chemosensitivity and that cells expressing low levels of FBW7, such as NCI-H1299 cells, have a mesenchymal phenotype. Furthermore, siRNA-mediated silencing or plasmid-mediated upregulation of FBW7 resulted in altered epithelial-mesenchymal transition (EMT) patterns in NSCLC cells. These data support a role for FBW7 in regulating the EMT in NSCLC cells. Conclusion: FBW7 is a potential drug target for combating drug resistance and regulating the EMT in NSCLC cells.

Keywords: FBW7 - NSCLC cells - cisplatin - epithelial-mesenchymal transition - drug resistance

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Introduction

Lung cancer has one of the highest incidences of all malignant tumors worldwide and is extremely harmful to human health. Approximately 80% of lung cancers are classified as non-small cell lung cancers (NSCLCs), which have a low 5-year survival rate that is less than 5% when accompanied by metastasis (Jemal et al., 2003). The pathogenesis of NSCLC is unclear and surgical treatment is the first choice for early-stage patients (Wozniak and Gadgeel, 2007). However, about 30-40% of NSCLC tumors cannot be effectively excised because they are diagnosed at an advanced stage. Therefore, chemotherapy is the main treatment for end-stage NSCLC patients. A cisplatin-based multidrug regimen is the standard NSCLC chemotherapy regime; however, drug resistance has become a bottleneck following the increased use of chemotherapeutics. Overcoming this bottleneck and thus solving drug resistance in NSCLC has become a hot topic in the field of lung cancer.

Many factors are involved in the development of drug resistance in lung cancers (Fruh, 2011; Cortot et al., 2013; Melguizo et al., 2013; Neel and Bivona, 2013). There is increasing evidence that the epithelial-mesenchymal transition (EMT) may be a key process regulating resistance to chemotherapy in malignant tumors (Hoshino et al., 2009; Kurrey et al., 2009). During the EMT, cell polarity and tight or adherent junctions are lost and cells acquire the ability to invade or migrate; this is recognized as a pathological process involved in tumor progression. The main markers of this process are a decrease or complete loss of the E-cadherin cell attachment protein and tight junction protein ZO-1, and increased expression of vimentin, aortic smooth muscle actin or fibronectin (Thiery, 2003; Thiery et al., 2009; Zeisberg and Neilson, 2009). There are some reports that NSCLC with an epithelial phenotype are more sensitive to chemotherapy than those with a mesenchymal phenotype (Thomson et al., 2005; Yauch et al., 2005) and that reexpression of E-cadherin in tumors with a mesenchymal phenotype restores chemosensitivity.

Many studies have investigated the relationship between the EMT and drug resistance in NSCLC and, although numerous mediators of EMT have been discovered (Maitah et al., 2011; Lee et al., 2012; Zhang et al., 2012; Byers et al., 2013), it has been difficult to

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reach a consensus. However, these studies suggest that identification of the key factor regulating the EMT could lead to an enhanced chemotherapeutic effect in cancer cells.

FBW7 (F-box/WD repeat-containing protein 7) is a member of F-box protein family located at 4q31 in humans. There are three subtypes of FBW7 (α , β and γ), which have different first exons (Spruck et al., 2002). FBW7 substrate binding occurs through a conserved phospho-motif known as the CDC4 phosphodegron which promotes ubiquitin ligase binding to the substrate, leading to substrate ubiquitination or proteasomal degradation (Grim et al., 2008). FBW7 substrates include many proteins involved in regulating cell growth and differentiation, including cyclin E, c-myc, c-Jun and Notch. Therefore, FBW7 regulates the cell cycle, cell growth and differentiation. There is evidence that FBW7 has a tumor suppressor function; deletion or mutation of the FBW7 gene has been reported in many different types of tumors (Calhoun et al., 2003; Cassia et al., 2003; Willmarth et al., 2004; Kwak et al., 2005; Song et al., 2008). Other studies indicate a link between FBW7 expression and chemoresistance (Inuzuka et al., 2011; Inuzuka et al., 2011; Wertz et al., 2011).

In this study, we aimed to investigate whether FBW7 mediates the EMT and controls chemosensitivity in NSCLC.

Materials and Methods

Cells and cell culture

Human NSCLC cell lines (A549, NCI-H1299 and HCC827) were purchased from the American Type Culture Collection (Manassas, VA, USA) and stored according to their instructions. All cells were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS (Gibco, Carlsbad, CA, USA) and 1% penicillin/streptomycin (Sigma, St. Louis, MO). All cells were maintained at 37°C in 5% CO_2 in a humidified incubator.

SiRNA-mediated silencing

Cells were transfected with FBW7 siRNA (Santa Cruz Biotechnology, sc-37547) using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Scrambled siRNA (Invitrogen) was used as a nonspecific control. The transfection medium was removed and replaced with culture medium 6 h after transfection. All experiments were performed 24 h after transfection and repeated three times.

Plasmids

FBW7 cDNA was amplified from A549 cells and subcloned into pcDNA3 vector. All the plasmids are verified by direct sequencing. FBW7 plasmid was transfected into cells by using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions.

Cell viability assay

Cell Counting Kit-8 (CCK8; Dojindo, Kumamoto, Japan) was used to measure relative cell viability after treatment. NSCLC cells (8×10^3 cells/well) were seeded

into 96-well plates and cultured for 24 h. The culture medium was then replaced by medium containing the indicated drug concentrations. After a further incubation of 48 h, 10 μ L/well of CCK-8 solution was added, cells were incubated for a further 4 h, and absorbance was measured at 450 nm using an MRX II microplate reader (Dynex Technologies, Chantilly, VA, USA). Relative cell viability was calculated as a percentage of untreated controls.

Western blotting

NSCLC cells transfected with FBW7 siRNA or FBW7 plasmid were washed twice in ice-cold phosphate buffered saline (PBS) and lysed using RIPA buffer (Sigma). Protein concentration was measured using a BCA Protein Assay kit (Thermo, IL, USA). Protein samples ($40 \mu g$ /lane) were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA) for Western blotting analysis. Membranes were probed with anti-E-cadherin, anti-vimentin and anti-FBW7 antibodies (Cell Signaling Technology) and protein expression was normalized to GAPDH expression (anti-GAPDH; Cell Signaling Technology). All antibodies were used at 1:1000 dilution.

Q-PCR analysis of FBW7 mRNA expression

NSCLC cells were cultured as described above. Total RNA was extracted with Trizol (Invitrogen), and cDNA was synthesized using a cDNA synthesis kit (TaKaRa, Japan) following the instructions provided by the manufacturers. The mRNA levels of FBW7 were measured by Q-PCR using the following gene-specific primer (Takara): forward, 5'-CACTCAAAGTGTGGGAATGCAGAGAC-3'; reverse, 5'-GCATCTCGAGAACCGCTAACAA-3'. The relative expression of FBW7 mRNA was normalized to β -actin expression using the following β -actin gene-specific primer (Takara): forward, 5'-GGAGATTACTGCCCTGGCTCCTA-3'; reverse, 5'-GACTCATCGTACTGCCTGGCTCCTA-3'; reverse, 5'-GACTCATCGTACTCCTGCTGCTG-3'.

Immunofluorescence

Cells were washed with ice-cold PBS, fixed in 4% paraformaldehyde for 30 min and incubated in 3% H₂O₂ for 15 min at 37°C. Non-specific binding was blocked by incubating cells with FBS for further 15 min. Following overnight incubation at 4°C with anti-E-cadherin or anti-vimentin (BioVision, California, USA) antibodies (all at 1:1000 dilution), the cells were washed with ice-cold PBS and incubated with the appropriate secondary antibody conjugated to goat anti-mouse horseradish peroxidase (at 1:2000 dilution; GE Healthcare, Piscataway, NJ, USA) for 1 h at room temperature. Cell nuclei were stained with DAPI (Sigma) and cells were observed using fluorescence confocal microscopy (Olympus, Japan).

Statistical Analyses

Data were analyzed using GraphPad Prism 5 software (GraphPad, San Diego, CA, USA) using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test., and are expressed as means \pm SEM. A *P* value < 0.05 was considered to be statistically significant.

Table 1. IC50 Values and Statistical Analyses forCisplatin Treatment in NSCLC Cell Lines

NSCLC cell line	Cisplatin IC50 (µg/mL)▲
A549	2.458 (2.330–2.639)
NCI-H1299	6.875 (6.018–7.733)*
HCC827	1.559 (1.407–1.710)*#

▲IC50 values indicate the cisplatin concentration [μ g/mL; mean (95% CI)]; **P* <0.05 vs. A549; **P*< 0.05 vs. NCI-H1299



Figure 1. FBW7 Expression Inversely Correlates with Cisplatin Chemosensitivity in NSCLC Cell Lines. (A) Western blotting shows differential FBW7 expression in A549, NCI-H1299 and HCC827 cells. (B) q-PCR showing the levels of FBW7 mRNA in all three NSCLC cell lines. (C) Analysis of cell viability showing differences in cisplatin chemosensitivity between all three NSCLC cell lines

Results

High levels of FBW7 expression are linked to increased chemosensitivity in NSCLC cells

To investigate the effect of FBW7 expression on cisplatin cytotoxicity in NSCLC cells, western blotting was used to measure FBW7 expression in three NSCLC cell lines: A549, NCI-H1299 and HCC827. A549 and HCC827 cells had much more higher levels of FBW7 expression than NCI-H1299 cells, which only had very low level expression (Figure 1A). Quantitative PCR (q-PCR) showed similar results for FBW7 mRNA levels (Figure 1B). Dose-dependent cell viability was then tested 48 h after cisplatin treatment alone using a CCK-8 assays. Increased cisplatin doses resulted in reduced cell viability in all three cell lines (Figure 1C). In addition, FBW7 high A549 and HCC827 were more sensitive to cisplatin than FBW low NCI-H1299 cells. The IC_{50} values for cisplatin in A549, NCI-H1299 and HCC827 cells were 2.485 µg/ mL (2.330-2.639 µg/mL), 6.875 µg/mL (6.018-7.733 µg/



Figure 2. FBW7 Upregulation Increases Cisplatin Chemosensitivity in NSCLC Cells. (A, B) Efficiency of siRNA-mediated FBW7 silencing in FBW7high A549 and HCC827 cell lines. (C) Efficiency of FBW7 overexpression in FBW7low NCI-H1299 cell lines. (D-F) Altering the levels of FBW7 in A549, HCC827 and NCI-H1299 cells by transfecting FBW7 siRNA or a FBW7 overexpression plasmid changed cisplatin chemosensitivity

mL) and 1.559 μ g/mL (1.407-1.710 μ g/mL; 95% CI), respectively (Table 1).

From the data above, we found that the level of FBW7 expression in NSCLC cells was correlated with cisplatin chemosensitivity, and higher FBW7 expression NSCLC cells were more sensitive to cisplatin, and NCI-H1299 cells which had low FBW7 level was resistant to cisplatin.

FBW7 upregulation increases cisplatin chemosensitivity in NSCLC cells

Our results suggested that high levels of FBW7 expression correlate with cisplatin chemosensitivity in NSCLC cells. In order to investigate the exact relationship between FBW7 and cisplatin sensitivity in NSCLC cells, we first used FBW7 siRNA to silence FBW7 in A549 and HCC827 which had more FBW7 expression. The efficiency of siRNA-mediated silencing at the protein level was assayed by western blotting (Figure 2A, B). We next used viability assays to investigate whether FBW7 silencing altered cisplatin chemosensitivity. FBW7 siRNA interference significantly attenuated cisplatin cytotoxicity in A549 and HCC827 cell lines and was accompanied by a considerable increase in the IC50 values to $4.244 \mu g/$

Table 2. IC50 Values for Cisplatin in NSCLC Lines after FBW7 Modulation

NSCLC		IC50 (µg/mL)▲		
	Control	FBW7 siRNA	FBW7 plasmid	
A549 NCI-H1299 HCC827	2.458 (2.330–2.639) 6.875 (6.018–7.733) 1.559 (1.407–1.710)	4.244 (3.908–4.580)* Not Determined 2.670 (2.326–3.013)*	Not Determined 2.112 (1.767-2.456)* Not Determined	

▲IC50 values indicate the cisplatin concentration [μ g/mL; mean, (95% CI)]; *P < 0.05 vs. control

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Figure 3. NSCLC Cells with an Epithelial Phenotype are More Sensitive to Cisplatin than Cells with a Mesenchymal Phenotype. Western blotting (A) and immunofluorescence (B) showing increased expression of epithelial marker proteins (E-cadherin) and reduced expression of mesenchymal marker proteins (Vimentin) in FBW7high A549 and HCC827 cells compared with FBW7low NCI-H1299 cells. (C) Twist silencing increases the expression of epithelial marker proteins (E-cadherin) and decreases the expression of mesenchymal marker proteins vimentin) in NCI-H1299 cells. (D) Twist silencing altered cisplatin chemosensitivity in NCI-H1299 cells

mL (3.908-4.580 μg/mL) and 2.670 μg/mL (2.326-3.013 μg/mL; 95% CI), respectively (Figure 2D, E; Table 2; *P* <0.05 vs. control).

We next used a FBW7 overexpression plasmid to upregulate FBW7 in NCI-H1299 cells which had very low FBW7 expression and measured the level of overexpression by western blotting (Figure 2C). We then measured the effect of FBW7 upregulation on chemosensitivity. We observed that FBW7 upregulation significantly increased cisplatin-induced growth inhibition in NCI-H1299 cells (Figure 2F) and was accompanied by a considerable reduction in IC50 values of 2.112 μ g/mL (1.767-2.456 μ g/mL) (Table 2; *P* <0.05 vs. control).

These data indicate that high expression of FBW7 is correlated with high cisplatin sensitivity in NSCLC cells.

NSCLC cells with an epithelial phenotype are more sensitive to cisplatin than cells with a mesenchymal phenotype

We next considered the possibility that whether NSCLC cells exhibiting an epithelial or mesenchymal phenotype may have a different response to chemotherapy. Western blotting revealed that cisplatin-resistant FBW7low NCI-H1299 cells had much lower E-cadherin expression but much higher vimentin expression than FBW7high A549 and HCC827 cells (Figure 3A).

Immunofluorescence analysis revealed similar results, with FBW7low NCI-H1299 cells demonstrating an almost complete absence of green E-cadherin staining and a much higher level of red vimentin staining than that observed in FBW7high A549 and HCC827 cells (Figure 3B).

Twist is generally acknowledged to be an important gene involved in triggering cancer cells to undergo the EMT (Moreno-Bueno et al., 2009). We therefore we used Twist siRNA to downregulate Twist and alter the EMT transition in FBW7low NCI-H1299 cell lines (Figure 3C). Twist siRNA led to increase epithelial marker E-cadherin

Table 3. IC50 Values and Statistical Analyses ofMesenchymal NCI-H1299 Treated with Cisplatinafter Twist Interference

NSCLC	IC50 (μg/mL)▲		
	Control	Twist siRNA	
NCI-H1299	6.875 (6.018–7.733)	2.362 (2.151-2.574)*	

▲IC50 values indicate the cisplatin concentration [μ g/mL; mean (95% CI)]; **P* < 0.05 vs. control

and decrease mesenchymal marker vimentin and also increase cisplatin chemosensitivity in this cell line, similar to that observed in cells overexpressing FBW7 (Table 3; Figure 3D).

These results show that FBW7low NCI-H1299 cells exhibit a mesenchymal phenotype, while FBW7high A549 cells and HCC827 cells exhibit an epithelial phenotype. Furthermore, we observed that epithelial-type NSCLC cells are more sensitive than mesenchymal-type cells to cisplatin.

FBW7 plays a role in regulating the EMT transition in NSCLC cells

We have demonstrated that altering FBW7 expression affects cisplatin chemosensitivity in different NSCLC cells, and showed a correlation between the EMT and cisplatin chemosensitivity in NSCLC cells. We therefore considered the possibility that FBW7 is involved in the EMT program. In order to test this, we transfected either the FBW7 expression plasmid or siRNA into mesenchymal FBW7low NCI-H1299 cells or epithelial FBW7high A549 and HCC827 cells, and measured the effects on EMT marker expression. FBW7low NCI-H1299 cells transfected with the FBW7 expression plasmid had increased E-cadherin expression (Figure 4A), indicating that FBW7 upregulation induced an epithelial phenotype in these cells. Immunofluorescence also showed that FBW7 upregulation in NCI-H1299 cells decreased vimentin expression (Figure 4A).

In addition, FBW7 silencing in FBW7high A549 and HCC827 cells reduced the expression of epithelial marker proteins and increased the expression of mesenchymal marker proteins, as shown by western blotting and immunofluorescence (Figure 4B, C).

FBW7 controls cisplatin sensitivity in NSCLC cells through regulating the EMT

TGF- β 1 is often used to induce the EMT. We therefore used TGF- β 1 to induce epithelial-type FBW7high A549 and HCC827 cells to undergo the EMT and investigated changes in both EMT marker expression and cisplatin sensitivity. After 48h exposure to 10 ng/mL TGF- β 1, obvious changes in EMT marker proteins were observed in both cell lines. Both type cells exhibited changes towards a mesenchymal phenotype, demonstrated by their increased expression of E-cadherin, along with decreased vimentin expression (Figure 4D, E). We then measured the effect of TGF- β 1 treatment on cisplatin chemosensitivity. TGF- β 1induced mesenchymal A549 and HCC827 cells showed reduced cisplatin sensitivity (Figure 4F). However, FBW7 overexpression in TGF- β 1-treated cells reversed the

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Figure 4. FBW7 Controls Cisplatin Sensitivity in NSCLC Cells Through Regulating the EMT. Western blotting and immunofluorescence showing changes in EMT marker protein expression after FBW7 silencing in (A) NCI-H1299, (B) A549, and (C) HCC827 cells. Western blotting and immunofluorescence showing the effect of TGF-β1 with or without FBW7 overexpression plasmid on the phenotype of epithelial FBW7high A549 cells and HCC827 cells (D, E). Changes in the chemosensitivity of A549 and HCC827 cells after TGF-β1 treatment either with or without FBW7 overexpression were determined using CCK-8 assays (F)

EMT, demonstrated by reduced vimentin expression and increased E-cadherin expression (Figure 4D, E).

Discussion

The causes of drug resistance in lung cancer remain unclear (Fruh, 2011) although several mechanisms have been suggested to be involved (Fruh, 2011). Although some progress has been made, more work is necessary to overcome drug resistance.

FBW7 is reported to be a tumor suppressor and FBW7 deletions or mutations have been identified in many tumors (Mao et al., 2004). Decreased FBW7 expression has been shown to correlate with the generation and development of tumors and poor prognosis. In addition, FBW7+/- mice are more susceptible to radiation-induced tumorigenesis than FBW7+/+ mice (Zhang and Koepp, 2006). Moreover, FBW7 overexpression has an anti-tumor effect, and tumor growth is suppressed following FBW7 upregulation in tumor cells (Nash et al., 2001; Zhang and Koepp, 2006; Grim et al., 2008).

FBW7 is a member of the F-box family of proteins, which are substrate recognition components of the SCF multisubunit ubiquitin ligase that mediates the ubiquitindependent proteolysis of several oncoproteins, including cyclin E1, c-Myc, c-Jun, and Notch (Nateri et al., 2004, Akhoondi et al., 2007, O'Neil et al., 2007, Anzi et al., 2008, Bonetti et al., 2008, Kitagawa et al., 2009). Other reports suggest a link between FBW7 expression and drug resistance (Inuzuka et al., 2011; Inuzuka et al., 2011; Wertz et al., 2011). Wertz et al. suggested that profiling the FBW7 and MCL1 status of tumors, in terms of protein levels, messenger RNA levels and genetic status, could be useful for predicting the patient response to anti-tubulin chemotherapeutics (Wertz et al., 2011). Other studies showed that FBW7 deletion in T-cell acute lymphoblastic leukemia contributes to gamma-secretase inhibitor resistance (O'Neil et al., 2007; Thompson et al., 2007).

The EMT is an established cause of drug resistance.

Following the EMT, cancer cells can acquire the ability to proliferate, invade, migrate and evade apoptosis; they are also less sensitive to chemotherapeutics. The EMT has been widely studied in NSCLC. Deletion or reduced expression of the epithelial marker E-cadherin is recognized as an important marker of EMT progression in NSCLC, and is a poor prognostic index (Thiery, 2002; Grunert et al., 2003; Nozawa et al., 2006). In this study, we demonstrated that FBW7low NCI-H1299 cells exhibit a mesenchymal phenotype, while FBW7high A549 and HCC827 cells exhibit an epithelial phenotype. We further showed that mesenchymal FBW7low NCI-H1299 cells are less sensitive to cisplatin than FBW7high A549 and HCC827 cells. In addition, our data indicate that FBW7 upregulation in NCI-H1299 cells reduced expression of the mesenchymal marker vimentin, increased expression of the epithelial marker E-cadherin, and enhanced cisplatin sensitivity. Our data provide evidence that FBW7 plays an important role in regulating drug resistance in NSCLC cells. Reexpression of FBW7 in drug-resistant NSCLC cells may therefore reverse the EMT and therefore enhance cisplatin cytotoxicity.

Our data support the view that FBW7 is a tumor suppressor, and demonstrates that deletion or reduced expression of FBW7 plays an important role in regulating the EMT in NSCLC cells, may promote tumor cell invasion and migration, and result in drug resistance. This finding not only supports the use of FBW7 as an prognostic marker in NSCLC patients but may also provide a new approach for treating NSCLC.

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