

Article

FBXW7 Acts as an Independent Prognostic Marker and Inhibits Tumor Growth in Human Osteosarcoma

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Abstract: F-box and WD repeat domain-containing 7 (FBXW7) is a potent tumor suppressor in human cancers including breast cancer, colorectal cancer, gastric cancer and hepatocellular carcinoma. In this study, we found that the expressions of FBXW7 protein and mRNA levels in osteosarcoma (OS) cases were significantly lower than those in normal bone tissues. Clinical analysis indicated that FBXW7 was expressed at lower levels in OS patients with advanced clinical stage, high T classification and poor histological differentiation. Furthermore, we demonstrated that high expression of FBXW7 was correlated with a better 5-year survival of OS patients. Multivariate Cox regression analysis indicated that FBXW7 was an independent prognostic marker in OS. Our *in vitro* studies showed that FBXW7 overexpression inhibited cell cycle transition and cell proliferation, and promoted apoptosis in both U2OS and MG-63 cells. In a nude mouse xenograft model, FBXW7 overexpression

slowed down tumor growth by inducing apoptosis and growth arrest. Mechanistically, FBXW7 inversely regulated oncoprotein c-Myc and cyclin E levels in both U2OS and MG-63 cells. Together these findings suggest that FBXW7 may serve as a prognostic biomarker and inhibit tumor progression by inducing apoptosis and growth arrest in OS.

Keywords: FBXW7; osteosarcoma; prognosis; apoptosis; tumor growth

1. Introduction

Osteosarcoma (OS) is the most common primary malignant cancer in bone [1]. Previous studies showed that the incidence of OS is correlated with patient age [2]. The annual incidence of OS is 0.017% in children under 10 years old, and 0.082% in children 10 to 19 years old [2]. Emerging evidence has shown that major advances in diagnosis and treatment have improved the outlook for OS patients, but satisfactory curative effects have not yet been achieved. Therefore, it is important to explore new biomarkers for predicting the prognosis and improving the outcome of OS patients.

FBXW7 (F-box and WD repeat domain-containing 7), also called hCDC4, is a well-characterized F-box protein that acts as a key component of the SCF (SKP1-CUL1-F-box protein) E3 ligase complex to recognize and bind to target proteins for ubiquitination and proteasomal degradation [3]. FBXW7 is a potent tumor suppressor in human cancers and functions as a key factor in multiple cellular processes including cell proliferation, apoptosis, cell cycle progression, invasion and drug resistance [4]. FBXW7 suppresses the initiation and development of tumors by targeting specific substrates for ubiquitination and proteasomal degradation [3]. Previous studies have identified several target proteins of FBXW7, such as c-Myc [5], cyclin E [6], c-Jun [7], mTOR [8], MCL-1 (myeloid cell leukemia-1) [9], p100 [10], Notch-1 [11] and SREBP1 (sterol regulatory element binding protein-1) [12].

Loss of FBXW7 expression has been observed in several human cancers, such as hepatocellular carcinoma [13], gastric cancer [14], breast cancer [15], blood cancer [16], colorectal cancer [17] and pancreatic cancer [18]. Studies found that gene mutations in FBXW7 result in its inactivation, and the overall mutation frequency is approximately 6% in all human cancers [19]. Yakohori *et al.*, reported that low expression of FBXW7 was correlated with malignant clinicopathologic features and poor prognosis in gastric cancer [14]. FBXW7 modulates leukemia-initiating cell activity by regulating MYC stability [16]. Moreover, inactivation of FBXW7 led to accumulation of cyclin E and promoted tumor progression in human pancreatic cancer [18]. A prior study reported that somatic hCDC4 mutations were infrequent in OS, and were unlikely to play an important role in aneuploidy of this tumor [20]. However, the clinical significance of FBXW7 and its role in human OS remain poorly understood.

In this study, we found that low expression of FBXW7 is correlated with malignant clinicopathologic parameters in OS. We demonstrate that FBXW7 is an independent prognostic factor for predicting 5-year survival of OS patients. Furthermore, FBXW7 overexpression exerts anti-cancer effects in OS by suppressing cell growth and inducing apoptosis *in vitro* and *in vivo*. Mechanistically, FBXW7 negatively regulated c-Myc and cyclin E abundance in OS cells. Our results suggest that FBXW7 may be a potent prognostic marker and inhibits tumor progression in OS.

2. Results

2.1. Clinical Significance of F-Box and WD Repeat Domain-Containing 7 (FBXW7) Expression in Osteosarcoma (OS) Specimens

To determine the expression of FBXW7 in OS specimens, we evaluated the levels of FBXW7 expression in 68 collected OS tissues and 20 collected normal bone tissues using immunoblotting. The expression of FBXW7 was significantly lower in OS tissues than in normal bone tissues ($p < 0.05$, Figure 1A). Furthermore, we performed real-time PCR to examine levels of FBXW7 mRNA in 20 randomly selected OS tissues and normal bone tissues ($n = 20$). Quantitative analysis indicated that the level of FBXW7 mRNA in OS tissues was lower compared with that in normal bone tissues ($p < 0.05$, Figure 1B). The expression of FBXW7 was considered as either low ($n = 34$) or high ($n = 34$) according to a cutoff value that was defined as the median of the cohort. As shown in Table 1, clinical association analysis indicated that loss of FBXW7 protein expression in OS tissues was prominently correlated with several poor clinicopathologic features including advanced clinical stage ($p < 0.001$), high T classification ($p = 0.046$) and poor histological differentiation ($p = 0.006$). Thus, our results indicate that the expression of FBXW7 is down-regulated in OS tissues and reduced expression of FBXW7 is associated with poor prognostic features.

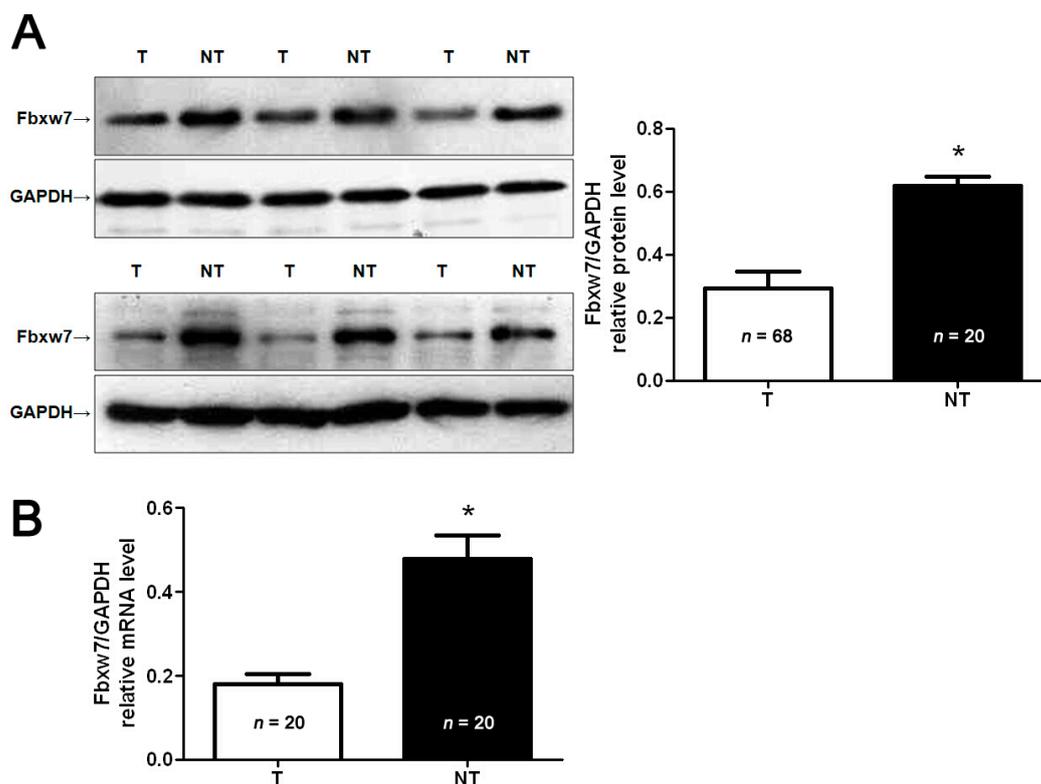


Figure 1. The expression levels of F-box and WD repeat domain-containing 7 (FBXW7) in osteosarcoma (OS) tissues and normal bone tissues. **(A)** Representative immunostaining showed the expression of FBXW7 protein in OS tissues (T) and normal bone tissues (NT). Quantitative data indicated that the expression of FBXW7 protein in OS tissues was lower than that in normal bone tissues; and **(B)** Comparison of differences in the expression levels of FBXW7 mRNA between OS tissues and normal bone tissues. * $p < 0.05$ by t test.

Table 1. Correlation between F-box and WD repeat domain-containing 7 (FBXW7) expression and the clinicopathological characteristics of the osteosarcoma (OS) patients.

Clinicopathologic Features		Total No. of Patients, <i>n</i> = 68	No. of Patients		<i>p</i>
			Low FBXW7	High FBXW7	
Gender	Male	40	19	21	0.622
	Female	28	15	13	
Age (years)	<24	49	24	25	0.787
	≥25	19	10	9	
Clinical stage	I	27	9	18	<0.001 *
	II	34	18	16	
	III	7	7	0	
T classification	T1	26	9	17	0.046 *
	T2	42	25	17	
M classification	M0	61	29	32	0.427
	M1	7	5	2	
Histology	Conventional osteosarcoma	58	28	30	0.493
	Others	10	6	4	
Histological differentiation	G1	27	8	19	0.006 *
	G2	41	26	15	

T, tumor; M, metastasis; * Statistically significant.

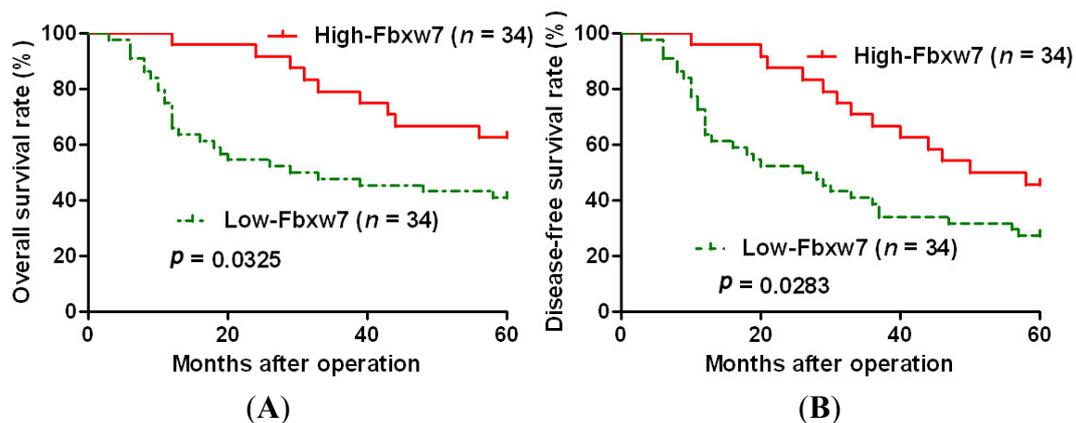


Figure 2. Prognostic significance of FBXW7 in OS cases. (A) Kaplan-Meier 5-year overall survival curves of OS patients according to the status of FBXW7 expression; and (B) Kaplan-Meier 5-year disease-free survival curves of OS patients according to the status of FBXW7 expression. The median expression value obtained for FBXW7 of the 68 OS samples detected by western blot was chosen as the cutoff value.

2.2. High Expression of FBXW7 Confers a Better 5-Year Survival for OS Patients

Next, we tested if FBXW7 expression status may have clinical significance on prognosis of patients with OS. Sixty-eight OS patients with clinical survival information (with a median follow-up time of 37.5 months) were analyzed by Kaplan-Meier estimation. Tumors with high expression of FBXW7

indeed associated with better overall survival and disease-free survival of OS patients ($p < 0.05$, Figure 2). Furthermore, multivariate Cox regression analysis indicated that FBXW7 expression was an independent factor for predicting both 5-year overall and disease-free survival in OS patients ($p = 0.030$ and 0.029 , respectively, Table 2). These data indicate that FBXW7 may act as a potent biomarker for predicting prognosis of OS patients.

Table 2. Multivariate analysis of various prognostic parameters in OS patients with Cox-regression analysis.

Variables	Overall Survival			Disease-Free Survival		
	HR	95% CI	<i>p</i>	HR	95% CI	<i>p</i>
Clinical stage	4.207	1.547–11.437	0.005 *	7.363	2.134–25.400	0.002 *
T classification	1.539	0.722–3.284	0.264	1.892	0.940–3.808	0.074
Histological differentiation	1.695	0.784–3.663	0.173	1.646	0.579–4.680	0.065
FBXW7 expression	2.976	1.109–7.987	0.030 *	2.327	1.093–4.955	0.029 *

HR, hazard ratio; CI, confidence interval; * Statistically significant.

2.3. FBXW7 Overexpression Inhibits OS Cell Growth and Promotes Apoptosis *in Vitro* and *in Vivo*

Previous studies demonstrated that FBXW7 acts as a tumor suppressor by inhibiting cancer cell proliferation and inducing apoptosis [3]. To identify the role of FBXW7 in OS, we transduced OS cell lines U2OS and MG-63 with empty vector (EV) retroviruses or FBXW7 retroviruses. Transfected cells were collected and subjected to BrdU assays and flow cytometry assays to evaluate cell cycle transition, proliferation and apoptosis. As measured by western blot, FBXW7 protein levels in both U2OS and MG-63 cells were significantly up-regulated by FBXW7 retrovirus infection ($p < 0.05$, Figure 3A). BrdU assays were performed to examine the effect of altering FBXW7 levels on tumor cell proliferation. We found that FBXW7 overexpression led to a significant reduction of cell proliferation in both U2OS and MG-63 cells ($p < 0.05$, Figure 3B). In both U2OS and MG-63 cells, the fraction of cells in G1 phase increased in the FBXW7 overexpression group compared with the control group. The percentage of cells in S phase decreased in the FBXW7 overexpression group compared with the control group ($p < 0.05$, Figure 3C). Furthermore, as determined by flow cytometry, the percentage of apoptotic U2OS and MG-63 cells were significantly elevated after FBXW7 overexpression ($p < 0.05$, Figure 3D).

We next implanted MG-63 cells into nude mice to examine if FBXW7 overexpression influenced tumor growth in mice and to explore the underlying mechanisms. MG-63 cells (5×10^6) expressing either EV or FBXW7 were implanted into nude mice via subcutaneous injection. Tumor growth curves revealed that FBXW7 overexpression significantly slowed down tumor growth in mice ($p < 0.05$, Figure 4A). We also performed immunohistochemistry for Ki-67 and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays in the xenografted tissues. Consistent with our *in vitro* data, FBXW7 overexpression inhibited proliferation and induced apoptosis *in vivo* ($p < 0.05$, Figure 4B,C). Thus, FBXW7 may exert an anti-cancer effect by inhibiting cell proliferation and inducing apoptosis in OS.

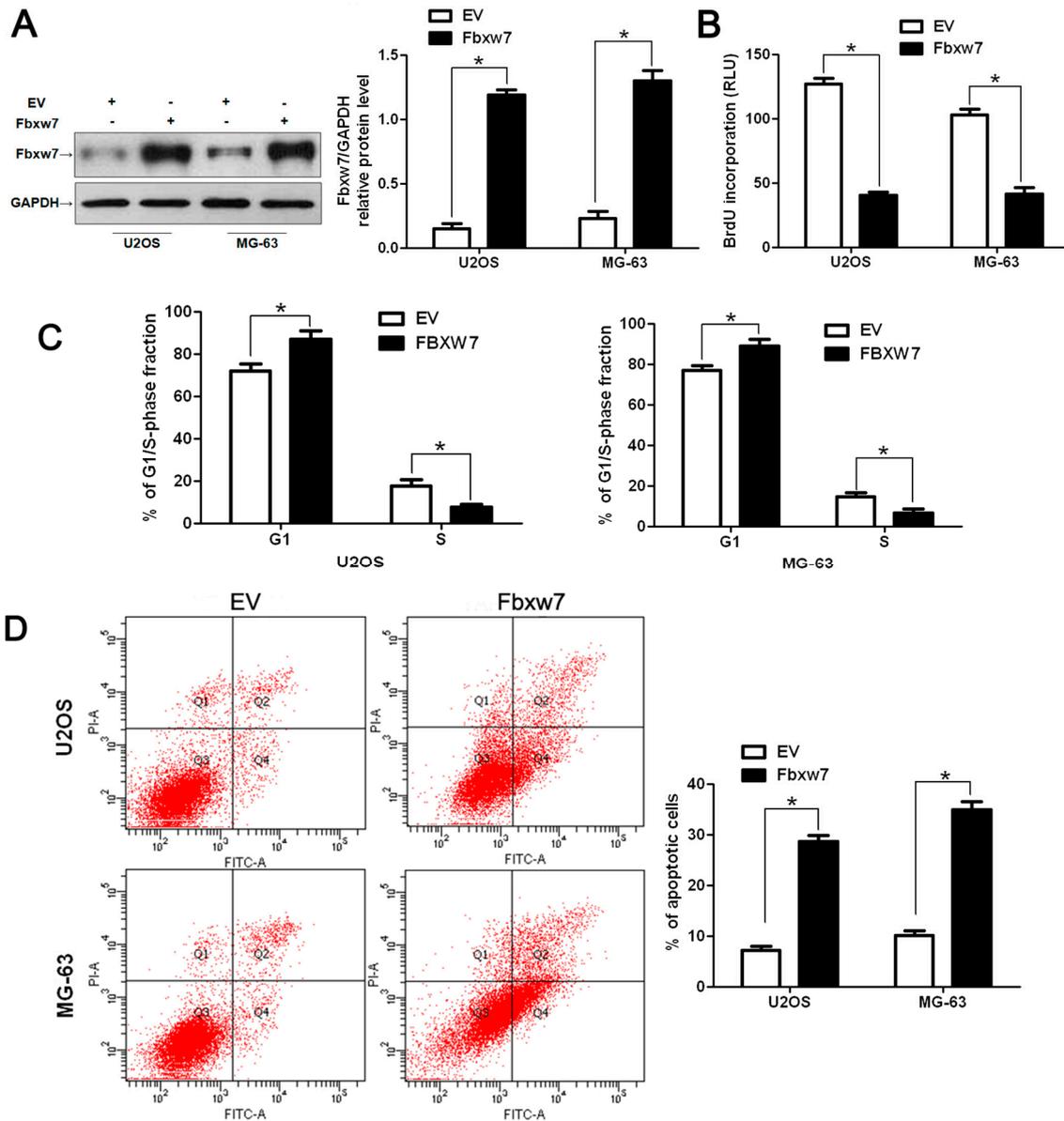


Figure 3. FBXW7 overexpression inhibits OS cell proliferation and induces apoptosis. (A) U2OS and MG-63 cells infected with empty vector (EV) and FBXW7 retroviruses were subjected to Western blot for FBXW7. $n = 6$; $* p < 0.05$ by t test; (B) Cell proliferation as measured by BrdU incorporation was inhibited by FBXW7 overexpression in U2OS and MG-63 cells compared with control cells. $n = 3$ repeats with similar results, $* p < 0.05$ by t test; (C) Cell cycle assay. The G1 phase fraction of FBXW7 overexpressing cells was increased compared with control cells. The S phase fraction of FBXW7 overexpressing cells was decreased compared with control cells. $n = 3$ repeats with similar results; $* p < 0.05$ by t test; and (D) Quantification of the apoptotic cell population by flow cytometry. FBXW7 overexpressing U2OS and MG-63 cells included a larger subset of apoptotic cells compared with control cells. $n = 3$ repeats with similar results; $* p < 0.05$ by t test.

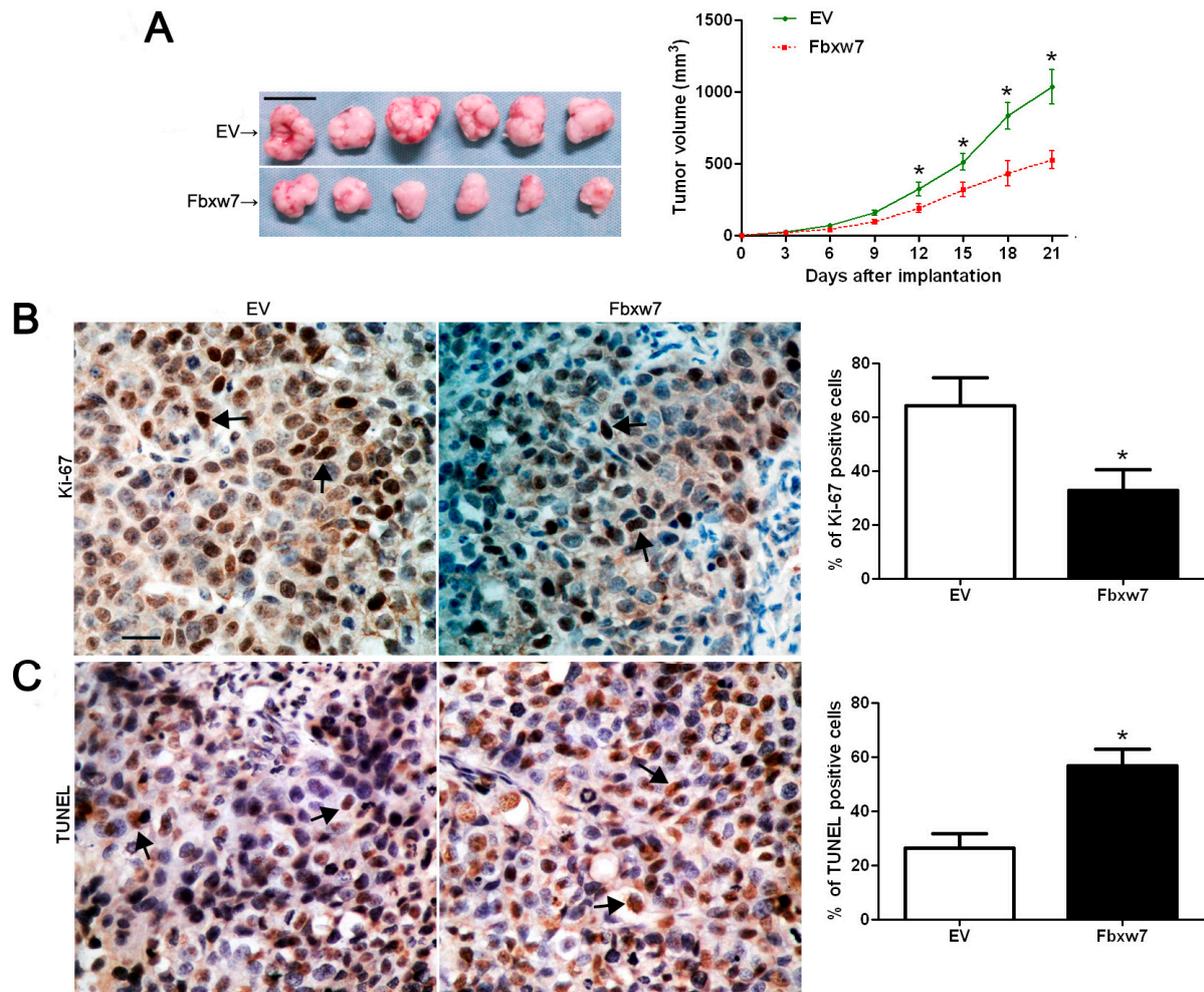


Figure 4. FBXW7 overexpression suppresses tumor growth in a nude mouse xenograft model. (A) Empty vector (EV)- or FBXW7-expressing MG-63 cells were implanted into nude mice via subcutaneous injection. Tumor growth curves indicated that FBXW7 overexpression slowed down tumor growth in mice. $n = 6$, * $p < 0.05$ by two-way ANOVA. Scale bar: 1 cm; (B) The average percentage of Ki-67-positive cells in tumors arising from the FBXW7 group was significantly lower than those in the control group. $n = 6$, * $p < 0.05$ by t test. Scale bar: 50 μm ; and (C) The average percentage of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive cells in tumors isolated from FBXW7 group was significantly higher than those in control group. $n = 6$, * $p < 0.05$ by t test. Black arrows indicate positive cells in each photomicrograph.

2.4. FBXW7 Inversely Regulates c-Myc and Cyclin E Abundance in OS Cells

Both c-Myc and cyclin E overexpression has been found in human OS, and FBXW7 targets both for degradation [5,6,21,22]. To determine whether FBXW7 regulates c-Myc and cyclin E protein levels in OS, FBXW7 overexpressing U2OS and MG-63 cells were subjected to western blot for c-Myc and cyclin E. As expected, FBXW7 overexpression decreased c-Myc and cyclin E protein levels in both U2OS and MG-63 cells ($p < 0.05$, Figure 5). Together these data suggest that FBXW7 may inhibit cell proliferation and induce apoptosis by targeting c-Myc and cyclin E degradation in OS cells.

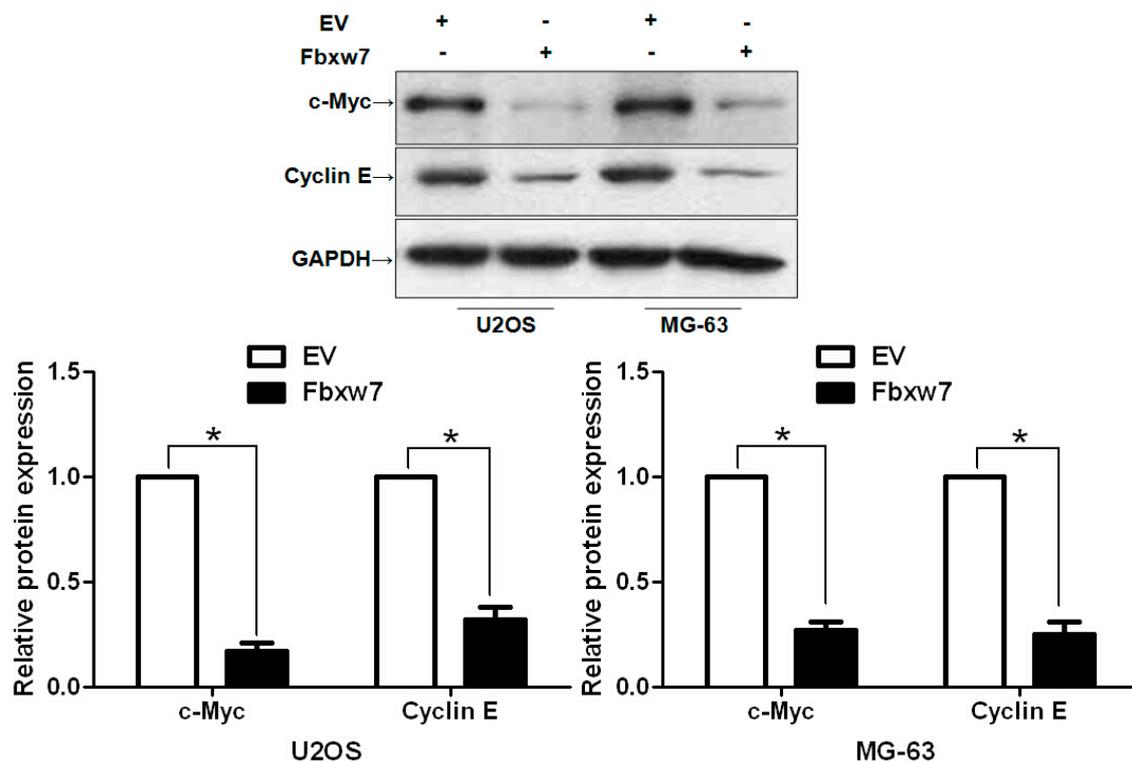


Figure 5. FBXW7 overexpression down-regulates c-Myc and cyclin E protein levels in OS cells. U2OS and MG-63 cells infected with empty vector (EV) and FBXW7 retroviruses were subjected to western blot for c-Myc and cyclin E. A representative western blot shows that FBXW7 overexpression reduced protein expression of c-Myc and cyclin E in OS cells. Data are representative of multiple repeated experiments with similar results; * $p < 0.05$ by t test.

3. Discussion

FBXW7 inhibits tumor growth by recognizing and promoting degradation of proteins involved in cell proliferation, cell cycle progression and apoptosis, including c-Myc, cyclin E and MCL-1 [3]. Increasing evidence has shown that aberrant low expression of FBXW7 is correlated with initiation and development of human cancer [4]. We initially detected FBXW7 expression status in 68 samples of surgical resected OS tissues and 20 collected normal bone tissues using western blot, and our data demonstrated that the expression levels of FBXW7 in OS tissues were significantly lower than those in normal bone tissues. Furthermore, FBXW7 protein was expressed at significantly lower levels in OS patients with advanced clinical stage, high T classification and poor histological differentiation. These results suggest that reduced expression of FBXW7 is correlated with poor prognostic features in OS, which is consistent with the status and clinical significance of FBXW7 in other human cancers [13–18]. Importantly, our data demonstrated that high expression of FBXW7 was correlated with a significantly better 5-year survival for OS patients. Multivariate Cox regression analysis indicated that FBXW7 was an independent factor for predicting both overall and disease-free 5-year survival in OS patients. Together, these results suggest that FBXW7 expression is critical for prognosis determination in OS patients.

Several studies have identified FBXW7 as a driving tumor suppressor in human cancers [13–18]. Mechanistically, FBXW7 exerts anti-cancer functions by inhibiting cell proliferation and inducing apoptosis [23,24]. In our study, we found that FBXW7 overexpression significantly inhibited cell cycle transition and proliferation and increased the subset of apoptotic cells in both U2OS and MG-63 cells. FBXW7 overexpression by retrovirus conferred an inhibitory effect on tumor growth in tumor-bearing mice. Furthermore, immunostaining for Ki-67 and TUNEL assays were performed on tumor nodules isolated from nude mice xenograft models. Percentages of Ki-67- and TUNEL-positive cells indicated that FBXW7 overexpression significantly reduced tumor burden by inducing apoptosis and growth arrest in the MG-63 tumor implantation model. Cyclin E1, which promotes cell growth by regulating cell cycle progression, is amplified and overexpressed in OS and its overexpression has potential prognostic and therapeutic implications [22]. c-Myc expression was found to be up-regulated in OS and c-Myc knockdown inhibited cell proliferation and induced apoptosis [21,25]. We found that FBXW7 overexpression decreased c-Myc and cyclin E protein levels in OS cells, suggesting that FBXW7 may act as a tumor suppressor by targeting c-Myc and cyclin E for degradation in OS.

In conclusion, here we show that FBXW7 is down-regulated in OS tissues and its low expression is associated with malignant clinicopathologic characteristics. Moreover, FBXW7 expression is an independent prognostic marker for predicting 5-year survival in OS patients. We demonstrate that FBXW7 overexpression reduces cell proliferation and induces apoptosis in OS cells, suggesting that FBXW7 may suppress tumor progression by inhibiting tumor growth. Furthermore, FBXW7 overexpression reduces c-Myc and cyclin E protein levels in OS cells. Taken together, we consider that FBXW7 may potentially act as a clinical biomarker, and may also be a therapeutic target, in OS.

4. Experimental Section

4.1. Ethical Review

The Shanghai Jiaotong University Ethics Committee approved all protocols according to the Helsinki Declaration (as revised in Tokyo 2004) and informed consent was signed by each patient. All animal protocols were approved by the Institutional Animal Care and Use Committee of Shanghai Jiaotong University (Shanghai, China).

4.2. Clinical Samples

A total of 68 paraffin-embedded OS and 20 normal bone tissue samples were obtained from the Department of Orthopaedic Surgery, Renji Hospital, School of Medicine, Shanghai Jiaotong University during the period from January 2006 to December 2008. All samples were used after obtaining informed consent (The Consent Identification Code: 2005-0036, 12 December 2005). The demographic features and clinicopathologic data are shown in Table 1. All specimens had confirmed pathological diagnosis and were classified according to the tumor-node-metastasis (TNM, AJCC, 6th edition) criteria.

4.3. Western Blot

The following primary antibodies were used in the immunoblotting assays: FBXW7 (WH0055294M2, Sigma, St-Louis, MO, USA), c-Myc (#5605, Cell Signaling Technology, Beverly,

MA, USA), cyclin E (#4129, Cell Signaling Technology) and GAPDH (G8140, US Biological, Salem, MA, USA). Horseradish peroxidase-conjugated secondary antibodies (Bio-Rad, Hercules, CA, USA) were used at a 1:1000–1:5000 dilution and detected using a Western Blotting Luminol Reagent (sc-2048; Santa Cruz Biotechnology, Santa Cruz, CA, USA), as described in our previous study [24].

4.4. Real Time Quantitative Reverse Transcription-PCR (qRT-PCR)

The following primers were used: FBXW7 sense primer 5'-AAAGAGTTGTTAGCGGTTCTCG-3' and antisense primer 5'-CCACATGGATACCATCAAAGT-3' and GAPDH sense primer 5'-CGGATTGGTCGTATTGG-3' and antisense primer 5'-TCCTGGAAGATGGTGATG-3'. The PCR amplification for the quantification of the FBXW7 and GAPDH mRNAs was performed using an ABI PRISM 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) and a SYBR[®] Premix Ex Taq™ II (Perfect Real Time) Kit (Takara Bio, Shiga, Japan), as previously reported [24].

4.5. Cell Lines and Transfection

The U2OS and MG-63 human OS cell lines (ATCC, Manassas, VA, USA) were cultured in complete Dulbecco's modified Eagle medium (DMEM, Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, Gibco) with 100 units/mL penicillin and 100 µg/mL streptomycin (Sigma) in a humidified 5% CO₂ incubator at 37 °C.

Retroviral vectors pMMP-FBXW7 were generated by inserting the FBXW7 cDNA into pMMP. Retrovirus packaging and transduction were described previously [23].

4.6. Cell Cycle, Proliferation and Apoptosis Detection

Flow cytometric analysis was made with fluorescence activated cell sorting (FACS) Calibur (Becton Dickinson, San Jose, CA, USA) and Cell Quest software (Becton Dickinson). For the proliferation assay, U2OS and MG-63 cells transfected with empty vector (EV) or FBXW7 were seeded into 96-well plates at 5000 cells/well for 24 h and assessed using a Cell Proliferation ELISA, BrdU (5-bromodeoxyuridine) (chemiluminescent) (Roche, Indianapolis, IN, USA). An Annexin-V-FLUOS Staining Kit (Roche) was used to analyze apoptosis levels as previously described [24].

4.7. In Vivo Experiments

Twelve female BALB/c nude mice (4–6 weeks old) were used to establish a nude mouse xenograft model. MG-63 cells (5×10^6) transfected with EV or FBXW7 were inoculated subcutaneously into the flank of each nude mouse ($n = 6$ mice each group). The tumor volume for each mouse was determined by measuring two of its dimensions and then calculated as tumor volume = length \times width \times width/2. After 21 days, the mice were sacrificed by cervical dislocation under anesthesia with ether and the tumor nodules were isolated for immunohistochemistry and TUNEL assays using a TUNEL assay Kit (4810-30-K, R&D Systems, Minneapolis, MN, USA) according to the manufacturer's guidelines.

4.8. Immunohistochemical Staining

Immunohistochemistry was performed on paraformaldehyde-fixed paraffin sections. Ki-67 (D2H10, #9027; Cell Signaling Technology) antibody was used in immunohistochemistry with streptavidin peroxidase conjugated (SP-IHC) method. Immunohistochemistry was performed as previously reported [23].

4.9. Statistical Analysis

Results are expressed as mean \pm SEM. Significance was established with the SPSS statistical package for Windows Version 13 (SPSS, Chicago, IL, USA) and GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA, USA), using a Pearson chi-squared test, the multi-variant Cox regression analysis, a two-tailed Student's *t* test, a Kaplan-Meier plot, a log-rank test or an ANOVA when appropriate. Difference were considered significant when $p < 0.05$.

5. Conclusions

In summary, this study shows that the expression of FBXW7 is reduced in OS tissues as compared with normal bone tissues and that low expression of FBXW7 is correlated with poor clinicopathological features in OS. Furthermore, we demonstrate that FBXW7 is an independent factor for predicting the overall 5-year survival and disease-free survival of OS patients. *In vitro* studies found that FBXW7 overexpression inhibits cell cycle transition and proliferation and induces apoptosis in OS cells. In tumor-bearing mice, FBXW7 overexpression slows down tumor growth with a reduced number of Ki-67-positive cells and an elevated number of TUNEL-positive cells. Otherwise, FBXW7 reduced the levels of c-Myc and Cyclin E in OS cells. Taken together, we suggest that FBXW7 may be a potent prognostic factor and a potential therapeutic target for OS.

Acknowledgments

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Author Contributions

Zhanchun Li and Jidong Zhang conceived and designed the experiments; Zhanchun Li, Jie Xiao, Kongzu Hu, and Jidong Zhang performed the experiments; Gang Wang and Maoqiang Li analyzed the data; Guangqi Cheng contributed reagents/materials/analysis tools; Zhanchun Li and Jidong Zhang wrote the paper.

Conflicts of Interest

The authors declare no conflict of interest.

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