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Enhancement of Cisplatin-Induced Apoptosis of Human Oral Squamous Cell Carcinoma by *FBXW7*

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Abstract

Background: About one-third of Oral Squamous Cell Carcinoma (OSCC) patients have a risk of occurrence and chemo resistance, making the survival abysmal. We aim to evaluate the role of F-Box/WD repeat-containing protein 7 (*FBXW7*) to further develop efficient treatment of chemo resistant OSCC.

Methods: *FBXW7* overexpression was induced by a lent viral vector, Lv-*FBXW7* or lv-NC (non-coding control) and overexpression efficiency was assessed using quantitative real-time PCR (qRT-PCR) and western blot of *FBXW7*. Cell viability was measured using MTT assay. The effects of *FBXW7* overexpression on cell migration and invasion was evaluated by the colony formation assay and Matrigel assay. Apoptosis of cells with lv-*FBXW7* transfection was measured by qRT-PCR and western blot analyses of BAX, BAK, MCL1 and BCL2 expression. Growth rate and Cisplatin sensitivity of CAL27 xenografts with or without *FBXW7* overexpression was monitored. Ki-67 and PCMA levels, which are biomarkers of intra-tumoral apoptosis, BAX, MCL1, Beclin1, LC3I and II, which are autophagy biomarkers, were assessed.

Results: Transfection of lv-*FBXW7* in SCC9 and CAL27 cells resulted in increased sensitivity to Cisplatin treatment, as evidenced by slower cell proliferation, lower colony formation and invasion, higher apoptosis and autophagy, compared to those transfected with lv-NC. Mice with CAL27 xenografts overexpressing *FBXW7* also demonstrated slower tumor growth and up regulation in Ki067 and PCNA. Tumors also showed higher apoptosis and autophagy activities.

Conclusion: *FBXW7* overexpression was herein shown to effectively sensitize OSCC cells to Cisplatin treatment *in vitro* and *in vivo*, potentiating *FBXW7* transduction as a new treatment strategy for OSCC.

Keywords: Oral squamous cell carcinoma; *FBXW7*; Chemo resistance; Cisplatin

Introduction

Oral Squamous Cell Carcinoma (OSCC) constitutes 90% cases of oral cancer, and is among the most prevalent cancers worldwide [1]. It is more common in males than females, but the number of female patients has gradually increased in recent years [2]. Surgery remains the main intervention for OSCC, but chemotherapy and radiotherapy are mostly used for those with advanced malignancy [3]. Due to the inability to detect OSCC early, one-third of OSCC patients are in advanced stage and have the risk of developing chemo resistant recurrent OSCC, resulting in a poor five-year survival rate of <60% [4]. Strategies to delay or block metastasis, and sensitize OSCC to chemotherapies, such as the front-line drug Cisplatin, are needed to effectively prolong the survival of patients.

F-Box/WD repeat-containing protein 7 (*FBXW7*) is 40 amino-acid protein that plays a critical role in regulating cytosolic misfiled proteins and damaged organelles mediates the ubiquitin-dependent proteolysis of several key regulatory proteins involved in cell division and cell fate determination, including c-Myc, cyclin E1, Notch, c-Jun [5-7]. *FBXW7* was first identified in budding yeast and the human *FBXW7* gene, located at chromosome 4q31q.3, is found to be deleted in 30% of cancers [8,9]. Such frequent deletion of *FBXW7* in cancer, which leads to an increase in genetic instability [10], a hallmark of human cancers, has intrigued many researchers because of its potential as a tumor suppressor. It's reported that *FBXW7* expression negatively correlates with the clinical grade of patients, making it a potential predictive marker [11]. By comparing the expression of autophagy biomarkers, including MCL1, BECN1 and ATG7, between OSCC tissues and adjacent normal tissues, it is found that the expression of MCL1 was significantly higher, while the expression of BECN1 and ATG7 mRNA was significantly lower, suggesting a decreased autophagy activity [12]. MCL1 mRNA expression also showed a significant negative correlation with *FBXW7* mRNA levels, while BECN1 and ATG7 mRNA expression was significantly positively correlated with *FBXW7* levels. In another work, overexpressing *FBXW7* in the OSCC cell lines and tumors was shown to inhibit cancer cell proliferation and promote autophagy [13]. Besides, the ability of *FBXW7* overexpression in enhancing temozolomide sensitivity in glioma has been demonstrated [14].

Based upon these findings on the anti-tumor role of *FBXW7*, we herein aimed to investigate the effects of *FBXW7* in enhancing the antitumor efficacy of Cisplatin on OSCC cells. We focused on evaluating if *FBXW7* overexpression using a lent viral vector could inhibit cancer proliferation, migration, and invasion and enhance OSCC's sensitivity to Cisplatin by increasing apoptosis and autophagy. The results of the

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study could potentiate the use of *FBXW7* overexpression to improve OSCC patients' survival.

Materials and Methods

Cell culture and cisplatin treatment

Human OSCC cell line CAL27, SCC9 was acquired from American Type Culture Collection (ATCC, Manassas, VA), and was cultured in RPMI-1640 medium with 10% fatal bovine serum (Gibco, Grand Island, NY) and 1% penicillin/streptomycin. Cells were cultured in a 37°C humidified incubator with 5% CO_2. For Cisplatin treatment, CAL27 cells transfected with Lv-*FBXW7* plasmids or control plasmids were treated with vehicle (PBS) or 4 μg/ml Cisplatin based on previously protocols [15].

Ectopic overexpression of *FBXW7*

Human *FBXW7* complementary DNA was reversely-transcribed from the longest transcript NM_013233 containing all three isoform-encoding sequences (GAGGATCCCCGGGTACCGGTCGCCACCATGAATC). The cDNA was inserted into the lent viral vector GV358 (purchased from Genechem, Shanghai, China) to create the complete functional overexpression plasmid named as Lv-*FBXW7*. Another non-coding control lent viral vector was also constructed as Lv-NC. Conditioned medium containing lent viruses was harvested 48 hours from transfected 293T cells and prepared for further transfection.

Animal studies

BALB/c nude mice of 4-6 weeks were subcutaneously injected with 5×10^6 CAL27 cells that were transfected with Lv-*FBXW7* expression plasmids in 100 μL PBS. The cells were divided into three groups, namely, control group, Cisplatin-treated group, and group transfected with *FBXW7* and treated with Cisplatin. Cisplatin treatment was started from the day after cell injection and the mice were intra-peritoneal injected with vehicle or 5 mg/kg body weight Cisplatin. The treatment was performed every 3 days from day 1 to day 28. Animal studies were approved by the institutional animal care and use committee of Changzhou Central Hospital.

RT-PCR

DNA was extracted using the TIZOL agent and reversely transcribed. The SYBR Green mix was used in real-time PCR. The following primers were used : *FBXW7*: forward: 5′-ACTGGGCTTGTACCATGTTCA-3′ and reverse: 5′-TGAGGTCCCCAAAAGTTGTTG-3′; GAPDH: forward: 5′-TGTTGCCATCAATGACCCCTT-3′ and reverse: 5′-CTCCACGACGTACTCAGCG-3′. MCL1: Forward: TGCTTCGGAAACTGGACATCA, Reverse: TAGCCACAAAGGCACCAAAAG; PCNA: Forward: GGCTCTAGCCTGACAAATGC, Reverse: GCCTCCAACACCTTCTTGAG; Ki67: Forward: AAGCCCTCCAGCTCCTAGTC, Reverse: TCCGAAGCACCACTTCTTCT; BAK: Forward: GTTTTCCGCAGCTACGTTTTT, Reverse: GCAGAGGTAAGGTGACCATCTC; BAX: Forward: CCCGAGAGGTCTTTTTCCGAG, Reverse: CCAGCCCATGATGGTTCTGAT; BCL2: Forward: GGTGGGGTCATGTGTGTGG, Reverse: CGGTTCAGGTACTCAGTCATCC.

Statistics

The data were shown as median (interquartile range). Data were analysed with ANOVA analysis (one- or two-way) with a post hoc test. Statistical significance was determined when p-value was less than 0.05.

Results

FBXW7 enhances cisplatin-induced inhibition of cell proliferation in OSCC

We first validated the overexpression of *FBXW7* in OSCC cells including SCC9 in Figures 1A-1C and CAL27 in Figures 1D-1F. The enhancing effects of *FBXW7* overexpression on Cisplatin treatment was investigated by MTT assay, which found that *FBXW7* was effective in retarding proliferation of OSCC cell lines (Figures 1G and 1H).

OSCC colony formation and invasion are further reduced in cisplatin-treated cells after *FBXW7* expression

Cell migration and invasion were assessed by the clonogenic assay and Matrigel cell invasion assay. Cells treated with Cisplatin together with *FBXW7* overexpression showed lower colony forming abilities (Figures 2A and 2B), and invasion (Figures 2C and 2D), indicating that *FBXW7* overexpression enhanced the effects of Cisplatin in inhibiting cell migration and invasion.

FBXW7 overexpression enhanced cisplatin-induced apoptosis in OSCC

The apoptosis of CAL27 cells was further evaluated in cells with overexpression of *FBXW7* and Cisplatin treatment by analyzing mRNA (Figures 3A-3D) and protein (Figures 3E-3I) levels of apoptosis biomarkers including BAX, BAK, MCL1 and BCL2. As shown in Figure 3A, it is evident that *FBXW7* overexpression enhanced the Cisplatin-induced apoptosis of CAL27 cells, evidenced by up regulation of BAX and BAK, and down regulation of MCL1 and BCL2.

FBXW7 overexpression sensitizes OSCC tumors to cisplatin treatment

The ability of *FBXW7* to enhance antitumor effect of Cisplatin on OSCC was next evaluated *in vivo*. Tumor volume was monitored for 4 weeks, after which tumor weight was measured after sacrificing the mice. While Cisplatin reduced tumor growth rate in $p<0.05$, Figure 4A and tumor weight of $p<0.05$, (Figures 4B and 4C). Compared to vehicle-treated mice, greater reduction was seen in mice with *FBXW7*-overexpressing tumors ($p<0.01$, compared to vehicle-treated mice). Our data suggested that *FBXW7* overexpression indeed led to lower tumor progression in Cisplatin-treated mice ($p<0.05$ compared to those without *FBXW7* overexpression). The reduced Ki67 Figure 4D and PCNA Figure 4E levels, also confirmed the enhancing effects of *FBXW7* overexpression in OSCC ($p<0.05$ compared to those without *FBXW7* overexpression).

FBXW7 enhanced cisplatin-induced apoptosis and autophagy of tumor tissues *in vivo*

We next evaluated the apoptosis and autophagy levels in the harvested tumor by measuring expression of BAX, MCL1 apoptosis markers, in Figures 5A-5C, Beclin1 and LC3I and II (Figure 5D-5F). Our data suggested that *FBXW7* amplified the Cisplatin-induced apoptosis and autophagy in OSCC tumor tissues.

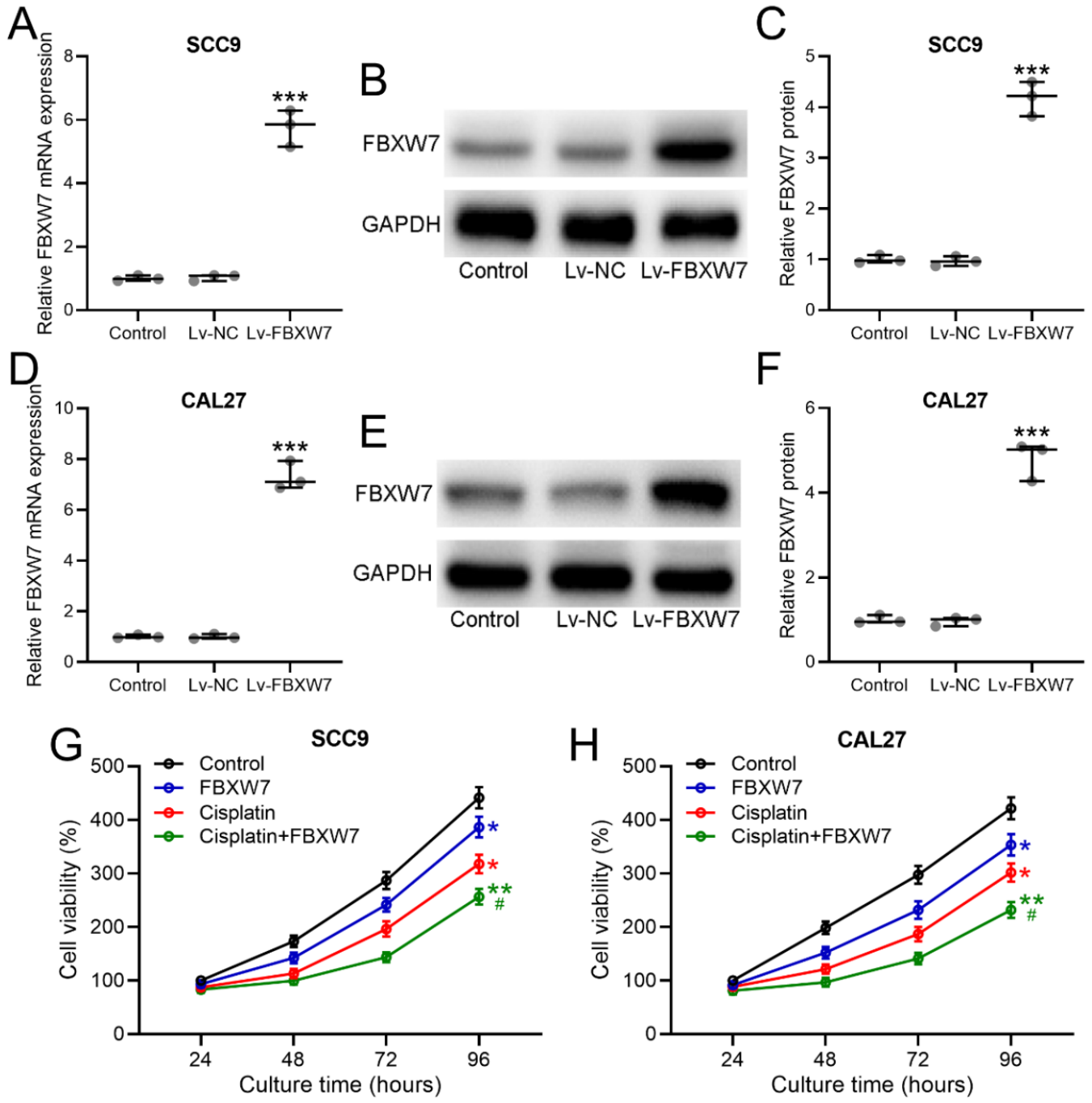

Figure 1. *FBXW7* enhanced Cisplatin-induced inhibition of cell proliferation in SCC9 and CAL27. SCC9 and CAL27 were transfected with LV-*FBXW7* or (lv-NC) negative controls for 48 h. qRT-PCR were used to measure the levels of *FBXW7* mRNA in SCC9 and CAL27 cells (A-D). Western blotting was used to measure the proteins levels of *FBXW7* in SCC9 and CAL27 cells (B and E). The expressions were normalized to control (C-F). SCC9 and CAL27 were transfected with LV-*FBXW7* and treated with vehicle or 4 μg/ml Cisplatin. At 24, 48, 72 and 96 hours after the transfection, cell viability was measured by MTT (G-H). The data were shown as median (interquartile range). **Note:** *p<0.05, **p<0.01, ***p<0.001 compared to control. #p<0.05 compared to Cisplatin group. **Note:** (—●—) Control, (—●—) *FBXW7*, (—●—) Cisplatin, (—●—) Cisplatin+ *FBXW7*.

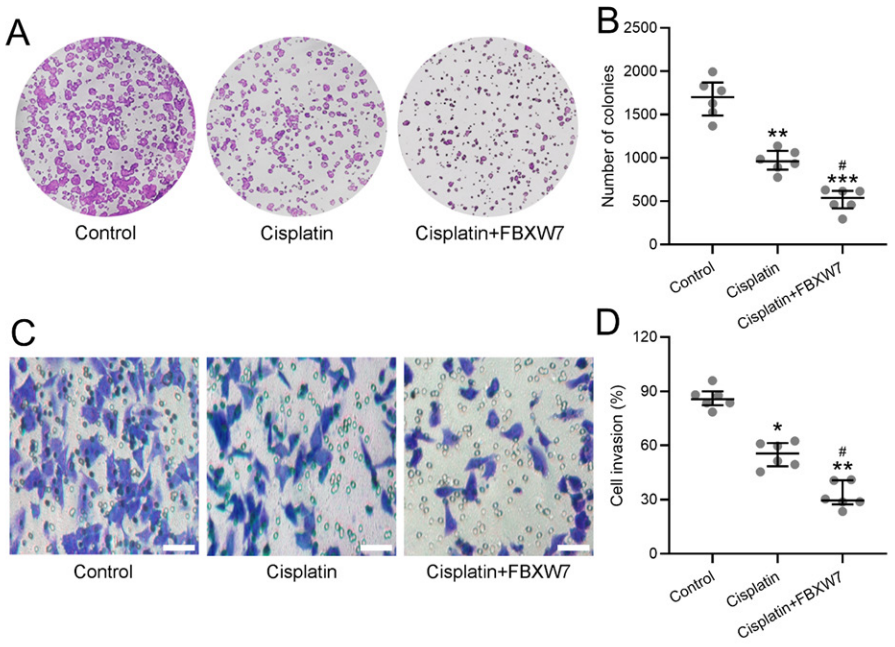

Figure 2. *FBXW7* enhanced Cisplatin-induced inhibition of cell proliferation and invasion of CAL27. CAL27 were transfected with LV-*FBXW7* and treated with 4 μg/ml Cisplatin. The colony formation assay was conducted 10 days after transfection (A) followed by counting colonies, (B). Cell invasion capabilities were measured 48 hours after treatment, (C) and the cell invasion ratio were calculated, (D) scale bar=50μm. The data were shown as median (interquartile range). **Note:** *p<0.05, **p<0.01, ***p<0.001 compared to control. #p<0.05 compared to Cisplatin group.

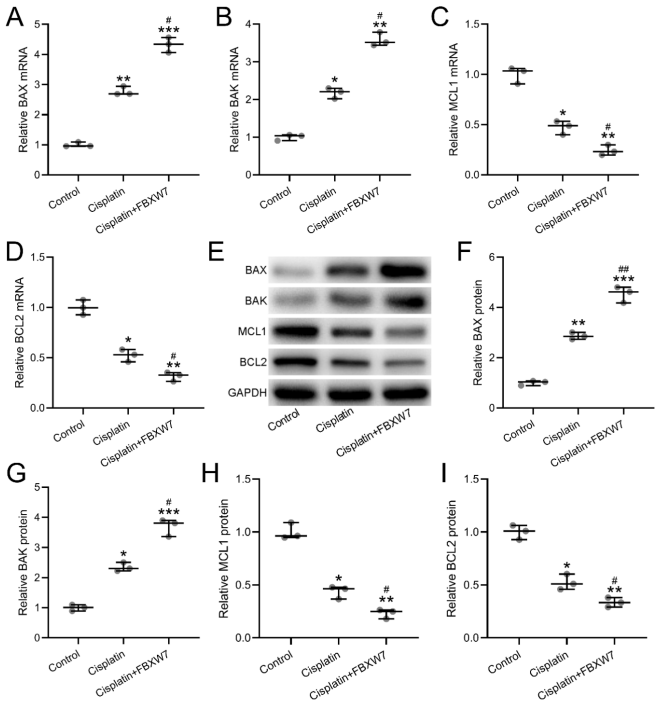

Figure 3. *FBXW7* enhanced Cisplatin-induced apoptosis of CAL27. CAL27 were transfected with LV-*FBXW7* and treated with 4 μg/ml Cisplatin for 48 hours. The mRNA levels of BAX, BAK, MCL1 and BCL2 were measured by RT-PCR (A-D). The protein level of proapoptotic molecules BAX, BAK and anti-apoptotic molecules MCL1 and BCL2 were examined by western blot (E). The expressions were normalized to control (F-I). The data were shown as median (interquartile range). **Note:** *p<0.05, **p<0.01, ***p<0.001 compared to control. #p<0.05, ##p<0.01 compared to Cisplatin group.

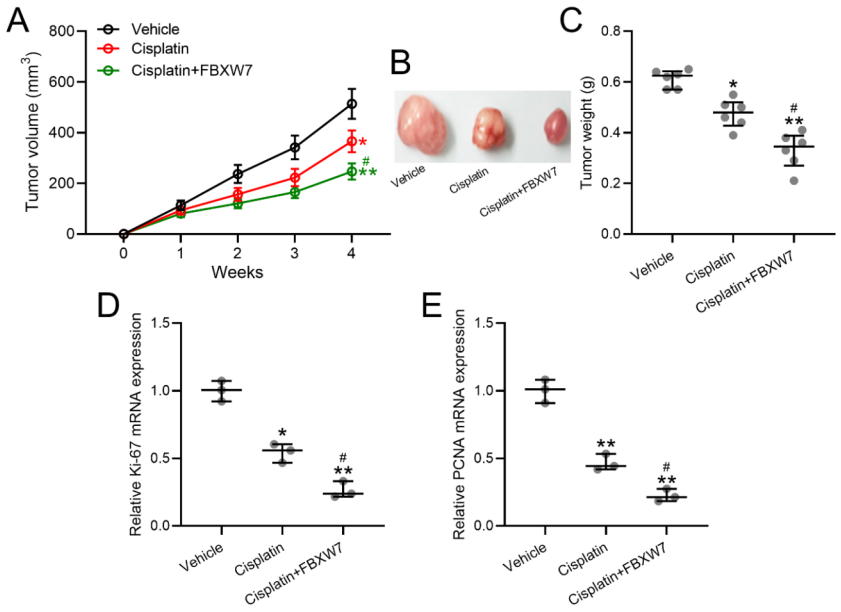

Figure 4. *FBXW7* enhanced Cisplatin-induced inhibition of cell proliferation of CAL27 *in vivo*. CAL27 xenograft tumor model was set up in Balb/c nude mice. CAL27 cells (5 × 10^6/mouse) transfected with and without Lv-*FBXW7* were injected subcutaneously into the flanks of the nude mice (n=6 in each group). Cisplatin or vehicle was intraperitoneally injected at 5 mg/kg b/w every 3 days from day 1 to 28. The tumor growth curve (A) and tumor weight at day 28, (B) were shown. Representative graph showing tumor growth at day 28, (C) qRT-PCR was used to measure the mRNA levels of Ki67 (D) and PCNA (E) in the tumor homogenate from each group. The data were shown as median (interquartile range). **Note:** *p<0.05, **p<0.01 compared to vehicle. #p<0.05 compared to Cisplatin group. Note: (-o-) Vehicle, (-o-) Cisplatin, (-o-) Cisplatin+ *FBXW7*.

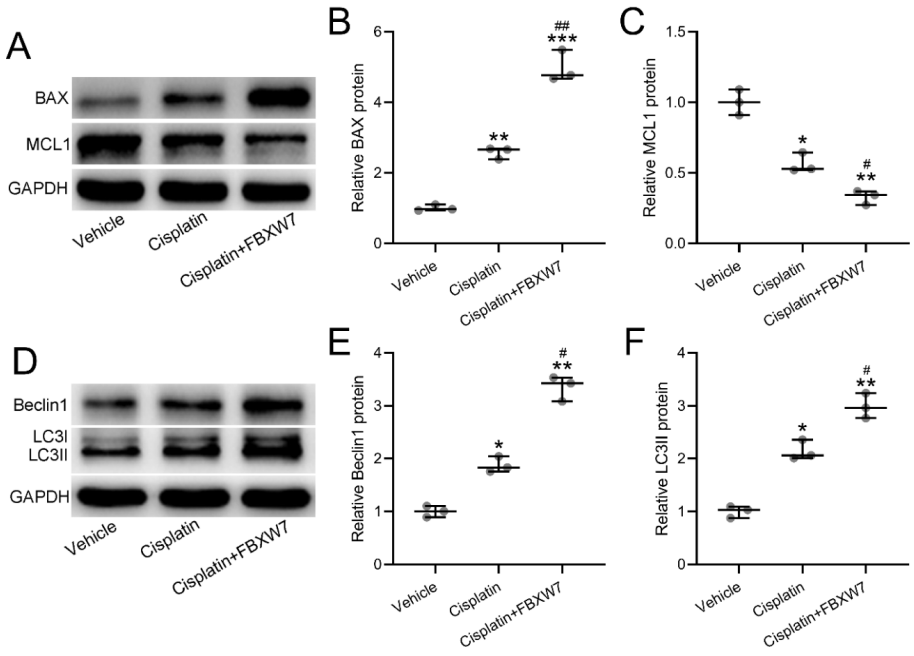

Figure 5. *FBXW7* enhanced Cisplatin-induced apoptosis and autophagy of tumor tissues *in vivo*. CAL27 xenograft tumor model was set up in Balb/c nude mice. CAL27 cells (5 × 10^6/mouse) transfected with and without Lv-*FBXW7* were injected subcutaneously into the flanks of the nude mice (n=6). Cisplatin or vehicle was intraperitoneally injected at 5 mg/kg body weight every 3 days from day 1 to 28. (A-D) The protein level of proapoptotic molecules BAX and anti-apoptotic molecules MCL1, autophagy related protein levels of Beclin1 and LC3 were examined by western blot. The expressions were normalized to control (B,C,E,F). The data were shown as median (interquartile range). **Note:** *p<0.05, **p<0.01, ***p<0.001 compared to vehicle. #p<0.05, ##p<0.01 compared to Cisplatin group.

Discussion

To overcome chemo resistance of OSCC, one of the important factors contributing to treatment failure, we explored *FBXW7* overexpression as a new strategy for sensitizing OSCC to Cisplatin treatment [16,17]. Our *in vitro* study showed that *FBXW7* overexpression was capable of suppressing cell proliferation, migration, and invasion, in cells with Cisplatin treatment. Such tumor-suppressing and Cisplatin-sensitizing function was further supported by *in vivo* study, which showed retarded tumor growth and enhanced tumor apoptosis and autophagy. These data suggest the significant clinical implication of *FBXW7* in OSCC.

Apoptosis, autophagy, and necrosis are three classic cell-death pathways, which are the mechanisms of cisplatin's anticancer efficacy. Our results showed that *FBXW7* promoted cell death by enhancing Cisplatin-induced cell death. In cancer, autophagy plays opposing roles as autophagy, through degrading organelles and cytoplasmic constituents, on the one hand inhibits tumorigenesis, and on the other hand induces metastasis and chemotherapy resistance. Anticancer drugs, including Cisplatin, are found to induce autophagy, a mechanism cancer cells exploits to acquire chemo resistance. Hence, autophagy has been pursued as a therapeutic target to develop novel anticancer drugs [18-20]. Several studies have investigated *FBXW7* as a regulator of autophagy in human diseases including in cancer [15,21-24]. In lung cancer, targeting *FBXW7* by miR-223 was shown inhibit Cisplatin-induced autophagy, which suppressed [15]. In studying hepatocarcinoma, *FBXW7* overexpression effectively inhibited sorafenib resistance through inhibiting autophagy [24]. Our findings corroborated *FBXW7* as not only a tumor suppressor but also an autophagy enhancer in OSCC, which contradicts the aforementioned studies, which could be explained by the complex role of autophagy.

Our study is not without limitation. Despite that *FBXW7* overexpression by directly transfecting lent viral vector is proven successful for *in vitro* study; such approach is not translatable as the delivery of *FBXW7* overexpressing vector to tumor cells is crucial for efficient gene transduction. However, with advances in gene delivery technologies, it is feasible to develop a gene vector, either viral or non-viral for targeted delivery of *FBXW7* plasmid to cancer. Further, the exact molecular mechanism of how *FBXW7* regulates autophagy remains to be elucidated. Exploring the effects of *FBXW7* in reducing Cisplatin resistance in animal models of advanced OSCC is also warranted.

Conclusion

In conclusion, by overexpressing *FBXW7* in OSCC, we showed that *FBXW7* is a tumor suppressor and can sensitize OSCC to Cisplatin treatment. *FBXW7* overexpression enhanced the effects of Cisplatin in inhibiting OSCC cell proliferation, migration and invasion *in vitro*, and also resulted in slower tumor growth and higher apoptosis and autophagy. Our data support further development of *FBXW7* overexpression as a potential therapeutic strategy for OSCC.

The control and role of the tumour suppressor FBXW7 in healthy and malignant cells have undergone significant advancement, although many unanswered problems still exist.

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Conflict of Interest

None declared.

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