FBXL10 contributes to the progression of nasopharyngeal carcinoma via involving in PI3K/mTOR pathway

Y. REN1,‡, L. WU‡, X. LI1, W. LI1, Y. YANG1, M. ZHANG1,*

1Department of Head and neck surgery, The Third Affiliated Hospital of Kunming Medical University, Tumor Hospital of Yunnan Province, Kunming 650118, China; 2Department of Radiology, The Second People's Hospital of Yunnan Province, Kunming 650021, China; 3Department of Radiation Oncology, The Third Affiliated Hospital of Kunming Medical University, Tumor Hospital of Yunnan Province, Kunming 650118, China

*Correspondence: zhangming977@126.com
‡Contributed equally to this work.
Received May 22, 2015 / Accepted July 22, 2015

Our study aimed to investigate whether F-box and leucine-rich repeat protein 10 (FBXL10) may play a pivotal role in nasopharyngeal carcinoma (NPC) development via involving in PI3K/mTOR pathway. We constructed an FBXL10 expression vector (pcDNA3.1-FBXL10). Then pcDNA3.1-FBXL10 and FBXL10-specific siRNA (siFBXL10) were transfected into human NPC cell line CNE1 and SUNE1 with Lipofectamine ® 2000. Moreover, cells were treated with PI3K inhibitor BEZ235. Besides, MTT assay and flow cytometry were respectively used to explore cell proliferation and apoptosis in vitro. Finally, the expression of key proteins involved in PI3K/AKT/mTOR pathway, such as P-AKT, AKT, P-P70, P70, P-Myc and Myc, were determined by western blot. Western blot analysis displayed that FBXL10 was overexpressed and suppressed after transfected by pcDNA3.1-FBXL10 and siFBXL10, respectively. Moreover, cell proliferation in FBXL10 overexpression group gradually increased compared with control group while obviously decreased in siFBXL10 group. Moreover, volume of apoptotic cells significantly increased with knockdown of FBXL10, which was similar with BEZ235 treatment. Besides, knockdown of FBXL10 decreased the expression levels of PI3K/mTOR pathway-related proteins, which was also similar with BEZ235 treatment. Notably, BEZ235 and siFBXL10 treatment induced significant increase of cell apoptosis and decrease of the expression levels of PI3K/mTOR pathway-related proteins than that only treated with siFBXL10. These findings indicate that FBXL10 may play a pivotal role in promoting cell proliferation and inhibiting cell apoptosis in NPC cells via targeting or functioning synergistically with PI3K/mTOR pathway. Knockdown of FBXL10 may be a novel therapeutic strategy for the treatment of NPC.

Key words: nasopharyngeal carcinoma, F-box and leucine-rich repeat protein 10, BEZ235, PI3K/mTOR pathway

Nasopharyngeal carcinoma (NPC) derived from epithelial cells is a high-incidence malignancy, particularly in Southern China [1]. Despite advances in surgical techniques, radiotherapy and chemotherapy, the prognosis of this disease is still poor [2, 3]. The epidemiologic evidence implies that genetic susceptibility, including tumor suppressor genes and proto-oncogenes, has important roles in the stages of NPC carcinogenesis [4]. However, several diagnostic and therapeutic targets have been identified in other cancers and remain not unclear in NPC [5, 6]. Thus, it is urgent to elucidate the molecular mechanism to detect the reliable therapeutic targets of this disease.

The F-box and leucine-rich repeat protein 10 (FBXL10, also known as JHDM1B, KDM2B and Ndy-1) belongs to the JmjC domain-containing histone demethylases and is considered as a tumor accelerator [7]. Penzo et al. demonstrated that FBXL10 expression might regulate cancer cell growth in a p53-dependent manner [8]. Tzatsos et al. confirmed that FBXL10 was markedly overexpressed in human pancreatic ductal adenocarcinoma (PDAC) and drive the pathogenesis of an aggressive subset of PDAC [9]. Kottakis et al. also verified that FBXL10 could function as a master regulator of Polycomb complexes and control cell self-renewal in breast cancer [10]. These findings suggest that FBXL10 contributes to the development of tumor, but regrettably, the roles of FBXL10 in NPC progression have not been fully discussed. Recently, the phosphatidylinositide 3-kinase/mammalian target of rapamycin (PI3K/mTOR) pathways have been shown
to play pivotal roles in tumor growth, counteract apoptosis, and enhance cell migration, thereby are considered as a key potential drug target for novel anticancer therapeutics [11-13]. Huang et al. demonstrated that the blockage of mTORC1 could enhance cell apoptosis in NPC and imply mTORC1 signal pathway as a potential target for NPC therapy [14]. Moreover, the dual PI3K/mTOR inhibitors, such as BEZ235 and PF-04691502, have been shown to induce apoptosis and inhibit cell growth in NPC, thus are evaluated as therapeutic drugs in preclinical models of NPC [15, 16]. Thus, PI3K/mTOR pathway may be a key mechanism involved in the progression of NPC. Considering the key roles of FBXL10 in tumor development, it can therefore be hypothesized that FBXL10 may play an important role in NPC progression via targeting PI3K/mTOR pathway.

In the current study, we constructed an FBXL10 expression vector (pcDNA3.1-FBXL10) and utilized FBXL10-specific siRNA to respectively promote and suppress the FBXL10 expression in human NPC cell line CNE1 and SUNE1. Moreover, cells were treated with BEZ235 to evaluate whether FBXL10 can target PI3K/AKT/mTOR pathway, which is previously shown to be a potent and highly selective reversible PI3K pathway inhibitor with antiproliferative and apoptotic activity in cancer [17]. Besides, the roles of FBXL10 in cell proliferation, apoptosis and activation of PI3K/AKT/mTOR pathway were respectively investigated. The objective of our study was to explore whether FBXL10 can play a role in NPC development, as well as whether the role is played via involving in PI3K/mTOR pathway. Our study will provide a new insight into the molecular mechanism of NPC progression and may help to develop novel targets for the treatment of this disease.

Materials and methods

Cell lines and cell culture. Human NPC cell line CNE1 and SUNE1 were purchased from the Cancer Research Institute of Sun Yat-sen University (Guangzhou, China) and reagents were purchased from Sigma (St Louis, MO, USA). The cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (RPMI-1640, Sigma-Alorich Co, St Louis, MO, USA) containing 10% heat-inactivated fetal bovine serum (FBS, Welgene Ltd.), penicillin (50 U/mL), and streptomycin (50 μg/mL), and then incubated in humidified incubator with 5% CO₂ at 37°C.

Vector construction and siRNA transfection. An FBXL10 expression vector (pcDNA3.1-FBXL10) was constructed by sub-cloning the full-length wild-type FBXL10 coding sequence into pcDNA3.1(+), and confirmed by sequencing. The pcDNA3.1-FBXL10 was transfected into cells while the empty construct pcDNA3.1 was transfected as a control. All the cell transfections were conducted using Lipofectamine 2000 reagent (Invitrogen) following the manufacturer's protocol. Stable FBXL10 transfecants were generated under G418 (Gibco, Paisley, UK) selection. To further explore the function of FBXL10 in CNE1 and SUNE1s, we used siRNA to silence its expression. The target sequence for FBXL10-specific siRNA (siFBXL10) was AAGUAAGUGAGACUGGAUCUCCACC, and control siRNA (no silencing) were synthesized by Geneharma Co (Shanghai, China). Then FBXL10-specific siRNA was transfected into cells. The effectiveness of siRNA silencing was assayed by Western blot analysis.

Cell proliferation assay. The cell proliferative capacity was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2Htetrazolium bromide (MTT) colorimetric assay. Detailed speaking, CNE1 and SUNE1 cells were respectively seeded on 96-well plates. Then MTT solution (Sigma, USA) was added to each well at 0 h, 24 h, 48 h, 72 h and 96 h after transfection. Each experiment was performed three times. After incubation for 1-4 h at 37°C, the cell proliferation/viability was calculated based on the absorbance at 570 nm which was measured by an enzyme-labeled instrument (Bio-Rad, USA).

Apoptosis analysis by flow cytometry. Cell apoptosis was detected by annexin V-Cy5 and PI staining (BioVision, K103-25) using FACS analysis. The cells were seeded culture dish and continued to incubate for 24 h. Part of cells in siFBXL10 group and control group were then pre-treated with PI3K inhibitor BEZ235 (1 μM) for 16 h while the other part of the cell was control. Cells in siFBXL10 group and control group were firstly washed with ice-cold phosphate-buffered saline (PBS) and resuspended in annexin V-binding buffer (10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, pH 7.4). Afterwards, annexin V-Cy5 (1:1,000) and 1 mg/ml propidium iodide (PI) solution was added to each. After incubation at room temperature (RT) for 5 min, the cells were determined at 488nm by a FACS Calibur flow cytometer (BD, USA) and analyzed with CELLQuest 3.0 software (BD, USA). In the scatter plot of double variable flow cytometry, the percentage of total apoptotic events (annexin V-Cy5 positive/PI negative) was defined as the sum of early apoptotic cells while the percentage (annexin V-Cy5 positive/PI positive) stands for late apoptotic cells.

Preparation of cell lysates and western blot analysis. The cells were washed with ice-cold PBS and lysed in Mammalian Protein Extraction Reagent (Sigma, C2978). Protein concentrations were examined by Bradford (Bio-Rad, Madrid, Spain). Protein samples with the same concentration per lane were separated on a 10-12% sodium dodecyl sulfate (SDS)-polyacrylamide gel. Then bands were blotted onto polyvinylidene difluoride membranes. After blocked in PBST (0.1% triton in PBS), the membrane was probed with primary antibodies overnight at 4°C. The membranes were then incubated with the appropriate horseradish peroxidase (HRP)- conjugated secondary antibodies (Santa Cruz Biotech). The immunoreactive protein bands were developed by enhanced chemiluminescence and analyzed by a densitometer. The following primary antibodies were used: P-AKT (S473), AKT, P-P70/S6K (T389), P70/S6K antibodies were obtained from Cell Signaling Technology (Beverly, MA). P-Myc and Myc antibodies were purchased from Roche Molecular Biochemicals. GAPDH antibody and horseradish peroxidase-conjugated second anti-
ROLE OF FBXL10 IN NPC PROGRESSION

body were purchased from Santa Cruz Co (Delaware Avenue, CA, USA). The relative expression level of these proteins was normalized based on the expression level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). To further quantify the results, blots were scanned and analyzed using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Statistics analysis. All experiments in our study were repeated three times and measurement data from multiple experiments were presented as the mean ± SD. Statistical analyses were performed using SPSS 17.0 statistical software. T-test (for two groups) or post-hoc Tukey test in one-way ANOVA (for more than three groups) was used to test the comparison between groups. A P-value of < 0.05 was considered to indicate a statistically significant result.

Results

Analysis of the expression level of FBXL10. As shown in Figure 1, the expression level of FBXL10 was determined by western blot. The results showed that FBXL10 in CNE1 and SUNE1 cells was overexpressed and suppressed in FBXL10 overexpression group and siFBXL10 group, respectively. Moreover, there were significant increased and decreased compared with control group (P < 0.05). However, after transfected with both pcDNA3.1-FBXL10 and siFBXL10, the expression level of FBXL10 was no significant difference compared with control (P > 0.05).

Analysis of proliferation rate. The proliferation abilities of CNE1 and SUNE1 cells in an experimental period of 48 h after transfection were measured by MTT assay (Figure 2). The results showed that the cell proliferation/viability of CNE1 cells in siFBXL10 group markedly decreased with the increase of treated time compared with blank group (P < 0.05). Meanwhile, the cell proliferation/viability of CNE1 cells in FBXL10 overexpression group gradually increased with the increase of treated time compared with blank group although the differences did not reach significance (P > 0.05). Similarly, the cell proliferation/viability of SUNE1 cells in siFBXL10 group obviously decreased with the increase of treated time compared with blank group while increased in FBXL10 overexpression group (P < 0.05).

Detection of cell apoptosis. The results of FACS analysis displayed that the percentage of apoptotic cells. As shown in Figure 3A, compared with cells in control group, the percentage of apoptotic CNE1 cells significantly increased after knockdown of FBXL10 by siRNA, treated by BEZ235, or both. Moreover, the percentage of apoptotic CNE1 cells significantly increased after treated with BEZ235 + siFBXL10 than only treated with siFBXL10 (P < 0.05). Besides, similar results were obtained in SUNE1 cells (Figure 3B).

Role of FBXL10 in PI3K/AKT/mTOR pathway. In order to further verify the relationship of FBXL10 with PI3K/mTOR pathway, the expression levels of key molecules involved in PI3K/AKT/mTOR pathway were determined by western
The result showed that the expression levels of P-AKT, P-P70 and P-Myc in BEZ235 group decreased compared to that in control group, and the expression levels of P-AKT, AKT, P-P70, P70, P-Myc and Myc in siFBXL10 group decreased. Meanwhile, the expression levels of all these proteins in BEZ235 + siFBXL10 group decreased than that in siFBXL10 group.

Discussion

PI3K/mTOR pathway is shown to be strongly involved in NPC development, and FBXL10 is identified as a key molecule in tumor development. However, the relationships of FBXL10 with PI3K/mTOR and NPC development remain unclear. In the present study, FBXL10 overexpression promoted cell proliferation both in human NPC cell line CNE1 and SUNE1 while knockdown of FBXL10 inhibited. Moreover, the percentage of apoptotic cells significantly increased with knockdown of FBXL10 by siRNA, which was similar with the results after treated by BEZ235. Besides, the expression levels of key proteins involved in PI3K/mTOR pathway decreased with knockdown of FBXL10, which also was similar with the results after treated by BEZ235. Notably, significant increase of cell apoptosis and marked decrease of the expression levels of PI3K/mTOR pathway-related proteins were observed after treated with BEZ235 + siFBXL10 than only treated with siFBXL10. Therefore, the role of FBXL10 and the relationship of FBXL10 with PI3K/mTOR pathway merit further discussion.

MTT assay of cell viability is a good method for assessing the proliferative effects of key factors [18]. In our study, we firstly used this method to evaluate the effects of overexpression and knockdown of FBXL10 on cell proliferation. The results showed that FBXL10 played a positive role in cell
proliferation in NPC cells and were in line with previous findings. For example, He et al. verified that FBXL10 was implicated in tumorigenesis and promoted cell proliferation in primary mouse embryonic fibroblasts [19]. Konuma et al. demonstrated that force expression of the FBXL10 could maintain self-renewal in hematopoietic stem cells [20]. In addition, FBXL10 is identified as an NF-kB-dependent anti-apoptotic protein, which could regulate tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induced apoptosis in various human cancer cells [21]. TRAIL may induce apoptosis in NPC cells via inhibiting the PI3K/Akt signaling pathway [22]. Moreover, Fukuda et al. also confirmed that FBXL10 deficiency accelerated neural progenitor cell death in embryonic neural development [23]. In our study, knockdown of FBXL10 contributed to the significantly increased percentage of apoptotic cells, indicating FBXL10 expression could inhibit cell apoptosis in NPC cells. Taken together, we speculate that FBXL10 may contribute to NPC development via promoting cell proliferation and inhibiting cell apoptosis.

In addition, BEZ235 is previously shown to be a potent inhibitor to selectively inhibit PI3K/mTOR pathway with apoptotic activity in NPC [24]. Our results showed that increased apoptotic cells after knockdown of FBXL10 was similar with the results after treated by BEZ235, suggesting that FBXL10 might play a role in NPC development via involving in PI3K/mTOR pathway. In order to further verify the relationship of FBXL10 with PI3K/mTOR pathway, we compared the expression levels of PI3K/mTOR pathway-related proteins after treated with BEZ235, siFBXL10 and both. Similar results were also obtained that knockdown of FBXL10 markedly decreased the expression levels of PI3K/mTOR pathway-related proteins, such as P-AKT, AKT, P-P70, P70, P-Myc and Myc, which was similar with the results after treated by BEZ235. Notably, BEZ235 and siFBXL10 treatment induced significant decrease of cell apoptosis and marked decrease of the expression levels of these proteins than that only induced by siFBXL10, implying that FBXL10 may play a key role in NPC development via targeting or functioning synergistically with PI3K/mTOR pathway.

Activation of AKT is believed to protect taxol-induced apoptosis in colon cancer cells via PI3K/AKT pathway [25]. Overexpression of P-AKT is thought to correlate with phosphorylation of epidermal growth factor receptor (EGFR) in NPC [26]. Zhu et al. verified that EGFR tyrosine kinase inhibitor AG1478 could inhibit cell proliferation and arrest cell cycle in NPC cells [27]. Moreover, the expression levels of P-P70 and P70 are analyzed to verify the growth-inhibitory activity of NVP-BGT226, a PI3K/mTOR dual inhibitor, against human head and neck cancer cells [28]. Esteva et al. demonstrated that the status of P-AKT and P-P70 correlated with trastuzumab response in patients with HER2-positive metastatic breast cancer, suggesting the potential of PI3K pathway activation as targeted therapies [29]. Besides, C-MYC is believed to function as an oncogene to stimulate cell proliferation, elicit apoptosis and change the sensitivity of cancer cells to chemotherapy [30]. Shepherd et al. observed that PI3K/mTOR inhibition may cause upregulation of c-MYC [31]. In our study, the expression level of these proteins decreased with knockdown of FBXL10 (Figure 4). It can therefore be speculated that FBXL10 may target or function synergistically with PI3K/mTOR pathway in human NPC cell lines. However, more functional evidences to verify whether FBXL10 contributes to NPC development via targeting or functioning synergistically with PI3K/mTOR pathway are still needed to be provided.

In conclusion, FBXL10 expression may play a pivotal role in promoting cell proliferation and inhibiting cell apoptosis in NPC cells via targeting or functioning synergistically with PI3K/mTOR pathway. Knockdown of FBXL10 may be a novel therapeutic strategy for the treatment of NPC.

Acknowledgements: Supported by Yunnan National Natural Science Foundation (No. 2011FB206).

References


