YWHAZ suppresses autophagy of osteosarcoma cells through interacting with TFEB

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Abstract

Tryptophan 5-monooxygenase activation protein zeta (YWHAZ) has been reported to play a crucial role in a variety of cellular processes. However, whether YWHAZ participated in the autophagy of osteosarcoma cells is unclear. In this study, our results showed that YWHAZ inhibited the autophagy of osteosarcoma cells through reduced the ratio of LC3-II/LC3-I and prevented the degradation of p62, two crucial markers of autophagy. Through interacting with YWHAZ, transcription factor EB (TFEB), a master controller of lysosomal biogenesis and an important regulator of autophagy, was remained in the cytoplasm and could not induce autophagy, verified by co-immunoprecipitation assay and nuclear and cytoplasmic extraction experiment. Taken together, our results reveale a new role of YWHAZ in regulating osteosarcoma autophagy and provided new clues for targeting YWHAZ.

Keywords

Osteosarcoma; YWHAZ; TFEB; autophagy.

1. Introduction

Osteosarcoma is highly malignant and can metastasize in the early stages of the disease. Most cases of osteosarcoma are sporadic, but certain environmental and genetic factors are associated with an increased risk of osteosarcoma[1,2]. Osteosarcoma is characterized by the production of osteoids and the propensity for metastasis, especially in the lungs[3]. At present, the main treatments for osteosarcoma include chemotherapy and amputation. However, there has been no significant improvement in the treatment of osteosarcoma in recent years. Thus, it is necessary to understand deeply the pathogenesis of osteosarcoma for finding new treatment strategies.

Autophagy is a relatively conservative process and regulates metabolic balance under stress conditions through liposomal degradation of damaged proteins and organelles[4]. Accumulating findings have shown that autophagy is widely involved in the development of tumors, such as tumor cell proliferation, metastasis, apoptosis, drug resistance and stem cell characteristics[5,6]. Therefore, studying the molecular mechanism of autophagy might provide new clues for osteosarcoma treatment. YWHAZ belongs to the 14-3-3 family, closely connected with the development of tumors[7]. YWHAZ has been reported to be a prognostic marker for various tumors and plays a crucial role in a variety of cellular processes, including proliferation, migration and invasion[8]. It has also been reported that YWHAZ inhibits autophagy in gastric cancer[9]. Whether YWHAZ participated in the autophagy of osteosarcoma cells is unclear.

TFEB acts as a master controller of lysosomal biological activity[10]. Overexpression of TFEB can increase the level of autophagy[11]. In this study, our results showed that YWHAZ inhibited the autophagy of osteosarcoma cells. Furthermore, TFEB was involved in this negative regulation of YWHAZ.

2. Materials and Methods

2.1 Antibodies and reagents

Anti-β-actin antibody, anti-p62 antibody, anti-TFEB antibody, HistoneH3 antibody, GAPDH antibody and anti-YWHAZ antibody were purchased from Proteintech Group, Inc. (Chicago, USA). Control rabbit IgG and LC3 antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2 Cell culture

Human osteosarcoma U2-OS cells were purchased from institute of life science Chinese Academy of Sciences (Shanghai, China) and cultured in McCoy's 5A medium (Sigma-aldrich, USA) with 10 % fetal bovine serum (PAN-Biotech, Germany) at 37°C, in a humidified atmosphere containing 5 % CO_2.

2.3 Construction of plasmid

FLAG-YWHAZ plasmid was constructed by amplifying the cDNA of human YWHAZ using PCR and inserted into PCMV-N-FLAG vector plasmid. The primer sequences of YWHAZ in the experiment are as follows: F: ATGGATCCATGGATAAAAATGAGCTG, R: ATGAATT CTTAA TTTTCCCCTCCTTC. The restriction endonucleases of BamHI and EcoRI were used. The PrimeSTAR® HS DNA Polymerase (TAKARA, Japan) was used for PCR system:

Component	Amount
Template plasmid	≤100 ng
Primer forward	1 μL
Primer reverse	1 μL
5 ×PrimeSTAR Buffer	10 μL
2.5 μM dNTP Mixtures	4 μL
PrimeSTAR HS DNA Polymerase	1 μL
ddH2O	up to 50 μL

Then, PCR reactions were performed according the following procedure

Temperature	Time	Cycle
95℃	2 min	1
95℃	30 sec	30
50-60℃	30 sec	
68℃	2 min / kb + 1 min	

Next, we extracted PCR product by TIANgel midi purification kit (TIANGEN, China).These PCR products and vector were double-digested according following reaction system, respectively. Then place tube in water of 37℃ for 4 hrs:

Component	Amount
Vector or insert	1 μg
10×K Buffer	2 μL
BamHI	1 μL
EcoR I	1 μL
ddH2O	up to 20 μL

Then the double-digested product was ligated by DNA blinting kit (TAKARA, Japan). The reaction system incubate overnight at 16℃.

Component	Amount
Double-digested Vector	100 ng / Cvector
Double-digested Insert	(500 ng × 4300 kb) / (1300 kb × Cinsert)
10×Ligation Reacton Buffer	2 μL
T4 DNA Ligase	1 μL
ddH2O	up to 25 μL

Next, we transformed the recombinant plasmid into DH5α and identified positive clones by colony PCR, and sent the positive clones to Shanghai Health Company for sequencing.

2.4 Small interfering RNA assays

The sense strand sequences of siRNA used in this study are as follows: YWHAZ siRNA: 5'-CCAAGGAGACGAAGCUGAATT-3', and control siRNA (NC): 5'-UUCUCCGA ACGUGU CACGU TT-3'. After transfection for 48 hours using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, the cells were harvested.

2.5 Cell transfection

For small interference RNA experiments, U2-OS cells were seeded sparsely 24 hours before transfection, and then transfected with siRNA (100 nM) or control siRNA according to the manufacturer's protocol. For overexpression experiments, U2-OS cells were transfected with the corresponding plasmids at the final concentration of 1.5 μg/mL using Lipofectamine 2000. After transfection for 48 hours, the cells were harvested.

2.6 Western blotting assay

U2-OS cells were transfected with the corresponding plasmids or siRNAs as indicated. Total cell lysate was harvested and prepared with buffer containing 20 mmol/L pH7.5 Tris, 150 mM NaCl, 1 % Triton X-100, sodium pyrophosphate, β-glycerophosphate, EDTA, Na_3VO_4, leupeptin, and 1 % protease inhibitor cocktail (Roche) . 20-50 μg of protein was separated by 10 % or 12 % SDS-PAGE. Blots were probed with antibodies against TFEB, YWHAZ, FLAG, β-actin or GAPDH, following the standard protocol.

2.7 Co-immunoprecipitation assay

U2-OS cells were cultured in 10 cm culture dishes and the cells were grown to a suitable density The cells were lysed in lysis buffer (1 mM NaF, 10 mM Na3VO4, 1 mM PMSF, 1mM protease inhibitor cocktail (Roche)) on ice for 30 min. The co-immunoprecipitation assay was performed with the protocols described[20] and the YWHAZ antibody and the control IgG were used. Approximately 1 mg of total protein from the lysate was mixed with 2 μg of YWHAZ antibody or IgG. Protein was pulled down with YWHAZ antibody, and the immune complexes were separated by Western blotting using antibodies against TFEB and YWHAZ.

2.8 Nuclear and cytoplasmic extraction

Nuclear and cytoplasmic extraction experiment was performed as follows. The harvested cells were re-suspended in 1 mL cold extraction buffer (10 mM Tris-HCl, pH 7.6, 10 mM KCl, 5 mM MgCl2) with protease inhibitors, 1 mM NaF, 10 mM Na3VO4, 1 mM PMSF, incubated on ice for about 10 min, and then lysed by addition of Triton X-100 to the final concentration of about 0.3% (w/v). 0.5 volume of nuclei isolation buffer (10 mM Tris-HCl, pH 7.6, 10 mM KCl, 5 mM MgCl2, 0.35M sucrose) was added to the extraction buffer, then nuclei were isolated by centrifugation at 600 g for 10 min. The membrane fraction containing mitochondria separated by centrifugation at 10000 g for 30 min. 4 volumes of acetone were added to cytoplasmic fraction to precipitate cytoplasmic proteins overnight at -20 ℃. Cytoplasmic proteins were gained by centrifugation at 10000 g for 30 min. The cellular components were mixed with SDS buffer for western blot assay.

3. Results

3.1 Construction and expression of FLAG-YWHAZ plasmid

Amplifying the cDNA of human YWHAZ using PCR and the amplified fragment of human YWHAZ cDNA using PCR was inserted into PCMV-N-FLAG vector plasmid (Figure 1A). Two restriction endonucleases of BamHI and EcoRI were used. The recombinant plasmid was verified by DNA sequencing. The expression of FLAG-YWHAZ plasmid in human U2-OS osteosarcoma cells was proved by Western blot (Figure 1B). This successfully constructed FLAG-YWHAZ plasmid was used to perform the following experiments.

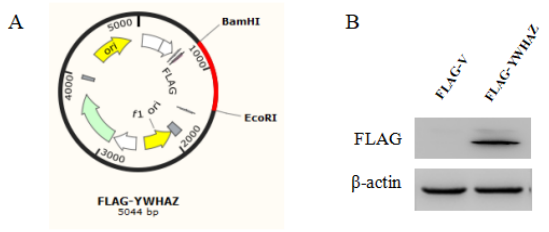

Figure. 1 The construction and expression of FLAG-YWHAZ plasmid. A: The model of plasmid construction. B: Expression of FLAG-YWHAZ plasmid in U2-OS

3.2 YWHAZ suppressed autophagy in osteosarcoma cells

YWHAZ play a critical role in a variety of cellular processes, such as cell cycle control, anti-apoptosis, metabolism, signal transduction, inflammation and cell movement[12]. Several lines of evidences showed the critical roles of YWHAZ in regulating autophagy in a variety of tumors[13]. To evaluate the effect of YWHAZ on autophagy in osteosarcoma cells, U2-OS cells were transfected with siRNA of YWHAZ to knockdown its expression or with FLAG-YWHAZ to overexpress this protein. The expressional levels of autophagy-related marker proteins, such as LC3-II and p62 were detected to test the occurrence of autophagy. As shown in Figure 2A, YWHAZ overexpression blocked the transition of LC3-I to LC3-II and reduced the ratio of LC3-II/LC3-I, suggesting the suppression of autophagy flow. Sequestosome 1 (SQSTM1 or p62), another marker of autophagy, normally acts as an autophagy receptor and can be simultaneously degraded with the cargo of autophagy[14]. Our result in Figure 2A showed that overexpression of FLAG-YWHAZ led to the increased level of p62, hinting the inhibition of autophagic degradation. On the contray, YWHAZ knockdown using its siRNA caused the increase -of LC3-II/LC3-I ratio, and the decreased level of p62 in U2-OS cells, suggesting that YWHAZ knockdown promoted the autophagy flow and degradation of p62. Taken together, these results indicated that YWHAZ inhibited the autophagy in osteosarcoma cells.

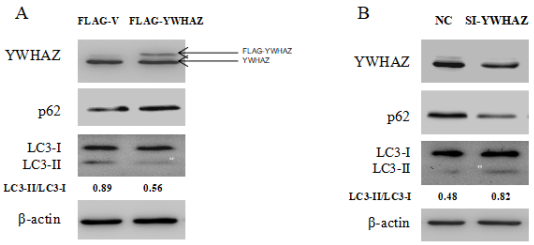

Figure. 2 The effect of YWHAZ on autophagy in osteosarcoma cells. A: Overexpression of YWHAZ, the protein levels of autophagy-related proteins, LC3 and p62 were detected. B: Knock down of YWHAZ, the protein levels of autophagy-related proteins, LC3 and p62 were detected.

3.3 YWHAZ negatively regulated autophagy through interacting with TFEB

Next, we investigated the possible mechanism of YWHAZ involved in autophagy of osteosarcoma cells. TFEB is known as a master transcription factor of autophagy, we then examined whether

YWHAZ interacted with TFEB in osteosarcoma cells. To this end, we performed an immunoprecipitation experiment in U2-OS cells using YWHAZ primary antibody. Our result in Figure 3 showed that TFEB was detected in the immune complex of YWHAZ primary antibody not in the control IgG group, suggesting the interaction of YWHAZ with TFEB protein. It is possible that YWHAZ negatively regulated osteosarcoma cell autophagy through binding to TFEB.

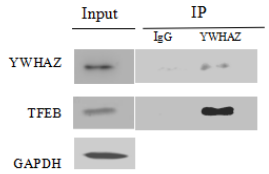

Figure. 3 The interaction of YWHAZ and TFEB. The YWHAZ antibody and the control IgG were used. Approximately 1 mg of total protein from the lysate was mixed with 2 μg of YWHAZ antibody or IgG. Protein was pulled down with YWHAZ antibody, the immune complexes were separated by Western blotting using antibodies against TFEB and YWHAZ.

3.4 YWHAZ prevented the nuclear localization of TFEB through binding to TFEB

TFEB is a master regulator of autophagy and lysosomal genes. Upon cellular stress, TFEB rapidly translocates to the nucleus to activate gene transcription[15]. We then tested whether YWHAZ was involved in the regulating of translocation of TFEB between cytoplasm and nucleus. For this purpose, U2-OS cells were knocked down YWHAZ using its siRNA and performed nuclear and cytoplasmic extraction assay. The result in Figure 4 showed that TFEB translocated from cytoplasm to nucleus after knockdown of YWHAZ, suggesting that YWHAZ prevented the nuclear location of TFEB through binding to TFEB.

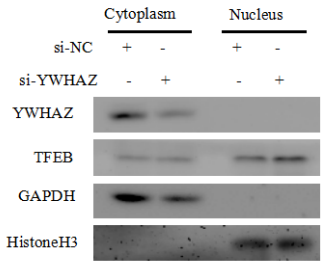

Figure. 4 Knockdown of YWHAZ promoted the nuclear translocation of TFEB. U2-OS cells were transfected with YWHAZ siRNA and performed nuclear and cytoplasmic extraction assay. The expressions of YWHAZ and TFEB were detected with standard western blot.

4. Conclusions

Osteosarcoma has a poor prognosis and metastasizes in the early stages of disease. The current main treatments remain chemotherapy combined with surgery, and there have been no significant improvements obtained in osteosarcoma treatments in recent years[16]. This pushed us to find new clues of osteosarcoma therapy through deeply investigating the pathogenesis of osteosarcoma. Currently, autophagy is the focus of life science research and widely involved in the development of tumors. Whether autophagy enhances or reduces the efficiency of osteosarcoma therapy has not been clearly determined. Here, we showed that YWHAZ suppressed the autophagy of osteosarcoma cells. YWHAZ as an intracellular regulator is related to various kinds of tumor functions, including tumor cell proliferation, apoptosis, and drug resistance[17]. YWHAZ has also been reported to inhibit autophagy in gastric cancer through PI3K/AKT/mTOR signaling pathway[18]. In this study, our results

showed that YWHAZ negatively regulated autophagy of osteosarcoma cells through binding to TFEB. TFEB transcription factor is a master autophagy regulator of autophagy. In normal conditions, TFEB was retained in the cytoplasm and couldn't promote the transcription of autophagy-related factors. Upon stress such as starvation and drugs treatments, autophagy was initiated and TFEB was released by its antagonistic partners in the cytoplasm and translocated to nucleus to promote transcription of autophagy-related factors[19]. Our results showed that YWHAZ knockdown promoted the nuclear location of TFEB, suggesting YWHAZ inhibited autophagy through retaining TFEB in the cytoplasm.

In conclusion, our study discovered a previously uncharacterized role of YWHAZ in the autophagy of osteosarcoma cells through modulating the nuclear translocation of TFEB. Therefore, our data indicate a new role of YWHAZ in regulating osteosarcoma autophagy, which will provide new ideas for targeting YWHAZ.

Acknowledgements

This work was partially supported by Natural Science Foundation of Guangdong Province (2016A030313420; 2018A030313544); Guangzhou Science and Technology Project (201707010263; 201804010066).

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