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ORIGINAL ARTICLE

FAT10 stimulates the development of osteosarcoma by regulating the JAK/STAT signaling pathway

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Summary

Purpose: *To investigate the potential function of FAT10 in the development of osteosarcoma (OS) and its mechanism.*

Methods: *Relative level of FAT10 in OS specimens and cell lines was detected by qRT-PCR. The correlation between FAT10 level and clinical features of OS patients was assessed by x^2 test. After intervention of FAT10 in MG-63 and U2OS cells, changes of FAT10 level, cell viability, clonality and proliferative capacity were respectively detected by qRT-PCR, CCK-8, colony formation and EdU assay. Moreover, dynamic change of FAT10 in OS cells induced with pro-inflammatory factors was examined by qRT-PCR. Protein levels of FAT10, p-STAT1, p-STAT3 and p-STAT5 in OS cells induced with TNF-a were determined by Western blot. The JAK2 inhibitor AZ960 was used to further confirm the role of the JAK signaling in FAT10-regulated development of OS.*

Results: *FAT10 was upregulated in OS specimens and cell lines, which was correlated to tumor size, WHO grade and distant metastasis of OS patients. Knockdown of FAT10 inhibited viability, clonality and proliferative capacity of MG-63 and U2OS cells. FAT10 was time-dependently upregulated in OS cells stimulated with IFN-γ and TNF-a, which was dose-dependently downregulated by the treatment of AZ960. Protein levels of FAT10, p-STAT1, p-STAT3 and p-STAT5 in OS cells induced with AZ960 were remarkably downregulated.*

Conclusion: *FAT10 is upregulated in OS samples, which stimulates the development of OS by activating the JAK/STAT signaling pathway.*

Key words: *FAT10, osteosarcoma, proliferation*

Introduction

Osteosarcoma (OS) is one of the most common primary bone malignancies in children and adolescents and is the third most commonly diagnosed primary bone malignancy in adults following chondrosarcoma and chordoma. The global incidence of OS is 3.4/1,000,000 [1]. Its prognosis is unsatisfactory even though great strides have been made on the diagnosis and treatment of OS in recent years, mainly because the rapid proliferation, high rates of early metastasis and chemotherapy resistance [2-4]. OS is prone to metastasize in an early phase. About 20% of initially diagnosed OS patients have metastases, involving 90% lung metastases. The 5-year survival of OS patients with distant metastasis ranges only 15-30% [5]. Therefore, a comprehensive understanding of the molecular mechanisms and pathogenesis of OS is beneficial to seek for novel therapeutic targets.

Human leukocyte antigen-F associated transcript 10 (FAT10) is an 18-kDa ubiquitin-like protein that serves as a signal of proteasome degradation [6,7]. FAT10 used to be considered as a mediator for cell growth and survival, and its change may induce abnormal cell growth that leads to carcinogenesis [8]. FAT10 is a multi-functional gene that participates in cell immunity, signal transduction,

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protein translocation and cell cycle progression [9]. It is reported that FAT10 is of great significance in cancer development and upregulated in several types of cancers [10-14]. FAT10 is mainly expressed in immune cells and tissues [15-18]. Relative level of FAT10 in non-immune cells can be induced by pro-inflammatory factors like IFN-γ, TNF-α and IL-6 [19]. The potential function of FAT10 in a tumorigenic inflammatory environment remains unclear.

In the present study, we aimed to explore the role of FAT10 in the pro-inflammatory environment of OS development, and the potential involvement of the JAK2 signaling pathway. Our findings provide novel ideas in guiding the diagnosis and treatment of OS.

Methods

OS specimens

A total of 62 pairs of OS and paracancer specimens that were surgically resected from OS patients who had completely recorded clinical and follow-up data were collected. Specimens were frozen in liquid nitrogen for 5 min, and stored at -80°C. Collection of tissue specimens for experimental use was obtained after informed consent and approval by the Ethics Committee of Qingdao West Coast New District People's Hospital.

Cell culture

Human OS cell lines (Saos-2, MG-63 and U2OS) and the osteoblast cell line (hFOB1.19) were obtained from Cell Bank, Chinese Academy of Sciences, Shanghai, China. Cells were cultured in Roswell Park Memorial Institute 1640 (RPMI 1640) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 mg/mL streptomycin (Invitrogen, Carlsbad, CA, USA) at 37°C, 5% CO_2. The medium was replaced every 2-3 days. Cell passage was conducted at 90% of confluence. Cells in the logarithmic growth phase were used in functional experiments.

Cell transfection

Transfection of shFAT10 #1, shFAT10 #2 or negative control (NC) (GenePharma, Shanghai, China) was conducted using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacture's recommendation.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), total RNAs were extracted from tissues or cells and reversely transcribed into complementary DNA (cDNA) using PrimeScript RT (TaKaRa, Tokyo, Japan). qRT-PCR was carried out using SYBR Green Kit (TaKaRa, Tokyo, Japan) with glyceraldheyde 3-phosphate dehydrogenase (GAPDH) as the internal reference. Primer sequences of FAT10 were 5'-GATGAGGAGCTGCCCTTGTT-3' (forward), and 5'-GCCTCTTTGCCTCATCACCT-3' (reverse).

Western blot

Total protein was extracted using radioimmunoprecipitation assay (RIPA) containing trypsin (Beyotime, Shanghai, China), and the concentration was detected using the bicinchoninic acid (BCA) method (Bio-Rad, Hercules, CA, USA). Protein sample was separated by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and loaded on polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA), which were incubated in tris-buffered saline-tween (TBST) containing 5% skim milk for 2 h. Membranes were immunoblotted with primary and secondary antibodies. Band exposure was achieved by chemiluminescent immunoassay, and grey values were analyzed using Gel-ProAnalyzer (United Bio), which were normalized to that of GAPDH.

Cell counting kit-8 (CCK-8)

Cells were seeded in 96-well plates with 1×10^3 cells suspended in 200 μL of medium per well. They were induced with 10 μl of CCK-8 solution per well at the indicated time points (Dojindo, Kumamoto, Japan). After cell culture for 2 h, absorbance at 450 nm was detected for plotting cell viability curves.

Colony formation assay

Cells were seeded in 6-well plates with 200 cells/well. After 2-week cell culture, colonies were fixed in paraformaldehyde, dyed with Giemsa and captured for counting.

5-Ethynyl-2'- deoxyuridine (EdU)

After 24-h cell culture, they were induced in 50 μmol/L EdU for 48 h, paraformaldehyde for 15 min and 0.2% glycine for 10 min. Cells were induced in 0.5% Triton X-100, dyed in Apollo in the dark for 30 min, and Hoechst in the dark for 10 min. Double phosphate buffered saline (PBS) washing was conducted between each procedure. Finally, cells were washed in 0.5% Triton X-100 for three times, and captured using a fluorescence microscope.

Statistics

SPSS 22.0 (IBM, Armonk, NY, USA) was used for statistical analyses. Data were expressed as mean±standard deviation. Differences between and among groups were compared by the t-test and one-way ANOVA, respectively and $p<0.05$ was considered as statistically significant.

Results

Upregulation of FAT10 in OS

In comparison with paracancer specimens, FAT10 was upregulated in OS specimens (Figure 1A). Consistently, FAT10 was upregulated in OS cell lines, especially in MG-63 and U2OS cell lines, and they were used in functional experiments (Figure 1B). A total of 62 OS patients were recruited, and they were subgrouped into high level group

and low level group with the median level of FAT10 in their OS specimens as the cut-off value. It was shown that larger tumor size, more advanced WHO grade and a higher rate of distant metastasis were detected in the high level group than in the other group ($p<0.05$) (Table 1). It was concluded that FAT10 was upregulated in OS cases, which could influence the tumor size, WHO grade and distant metastasis.

Intervention of FAT10 suppressed the proliferative capacity of OS

We constructed two FAT10 shRNAs and tested their transfection efficacy in the MG-63 and U2OS cells. Transfection of either of them could effectively downregulate FAT10 (Figure 2A). Knockdown of either shFAT10#1 or shFAT10#2 decreased viability of OS cells (Figure 2B,2C). Similarly, the clonality was weakened by knockdown of FAT10 (Figure 2D). EdU assay identically obtained the conclusion that knockdown of FAT10 suppressed the proliferative capacity of OS cells (Figure 2E).

FAT10 was regulated by the JAK/STAT signaling pathway

It is reported that IFN-γ and TNF-α can induce the active expression of FAT10 [20]. Here, the mRNA level of FAT10 was time-dependently upregulated in U2OS and MG-63 cells stimulated by IFN-γ and TNF-α (Figure 3A). To validate the involvement of the JAK signaling, OS cells were induced with an effective JAK2 inhibitor, AZ960. As qRT-PCR data revealed, the mRNA level of FAT10 was dose-dependently downregulated in AZ960-induced OS cells (Figure 3B). To avoid the off-target effect, the dose of 259 nM AZ960 was selected in

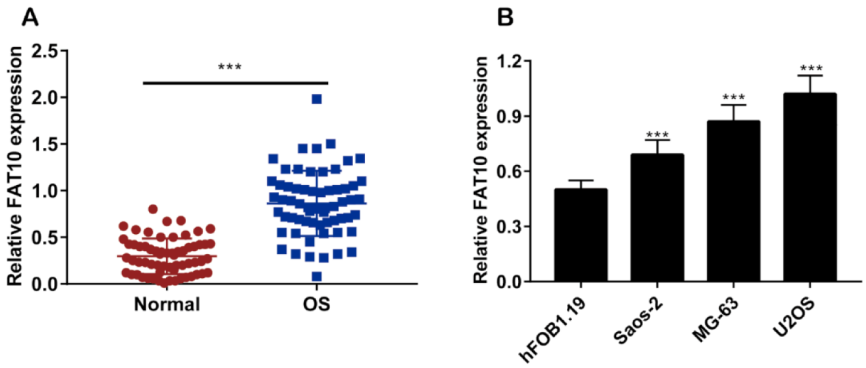

Figure 1. Upregulation of FAT10 in OS. **A:** Relative level of FAT10 in 62 pairs of OS specimens and paracancerous ones detected by qRT-PCR. **B:** Relative level of FAT10 in OS cell lines detected by qRT-PCR (***$p<0.001$).

Table 1. Correlation between FAT10 level and clinical features of OS patients (n = 62)

Clinicopathologic features	*Number of cases*	*FAT10 expression*		*p value*
		Low (n=30)	*High (n=32)*	
Age (years)				0.211
≤20	28	16	12	
>20	34	14	20	
Gender				0.779
Male	28	13	15	
Female	34	17	17	
Tumor size, cm				0.011
≤6	27	18	9	
>6	35	12	23	
WHO grade				0.023
I~II	30	19	11	
III~IV	32	11	21	
Distant metastasis				0.005
Yes	32	10	22	
No	30	20	10	

the following experiments. Here, doxycycline (Dox) was applied to inhibit the expression level of JAK2, aiming to prove that AZ690 inhibited the expression of FAT10 by downregulating JAK2, rather than the off-target effect. OS cells were pre-treated with the cytokine Ti for 24 h, followed by the treatment of Dox or not. Compared with Dox-free group, mRNA levels of JAK2 and FAT10 were remarkably lower in Dox-induced cells (Figure 3C). The above results indicated that JAK2 regulated FAT10 in OS cells.

JAK2 was the mediator of FAT10 induced by IL-6 and TNF-a

We have already proven that FAT10 could inhibit the proliferative capacity of OS with the involvement of JAK2. Compared with Ti-induced MG-63 cells without AZ960 treatment, those induced with Ti and AZ960 expressed a lower level of FAT10 (Figure 4A). The downregulation of FAT10 was more pronounced in AZ960-treated cells stimulated by both IL-6 and TNF-α (Figure 4B). It is

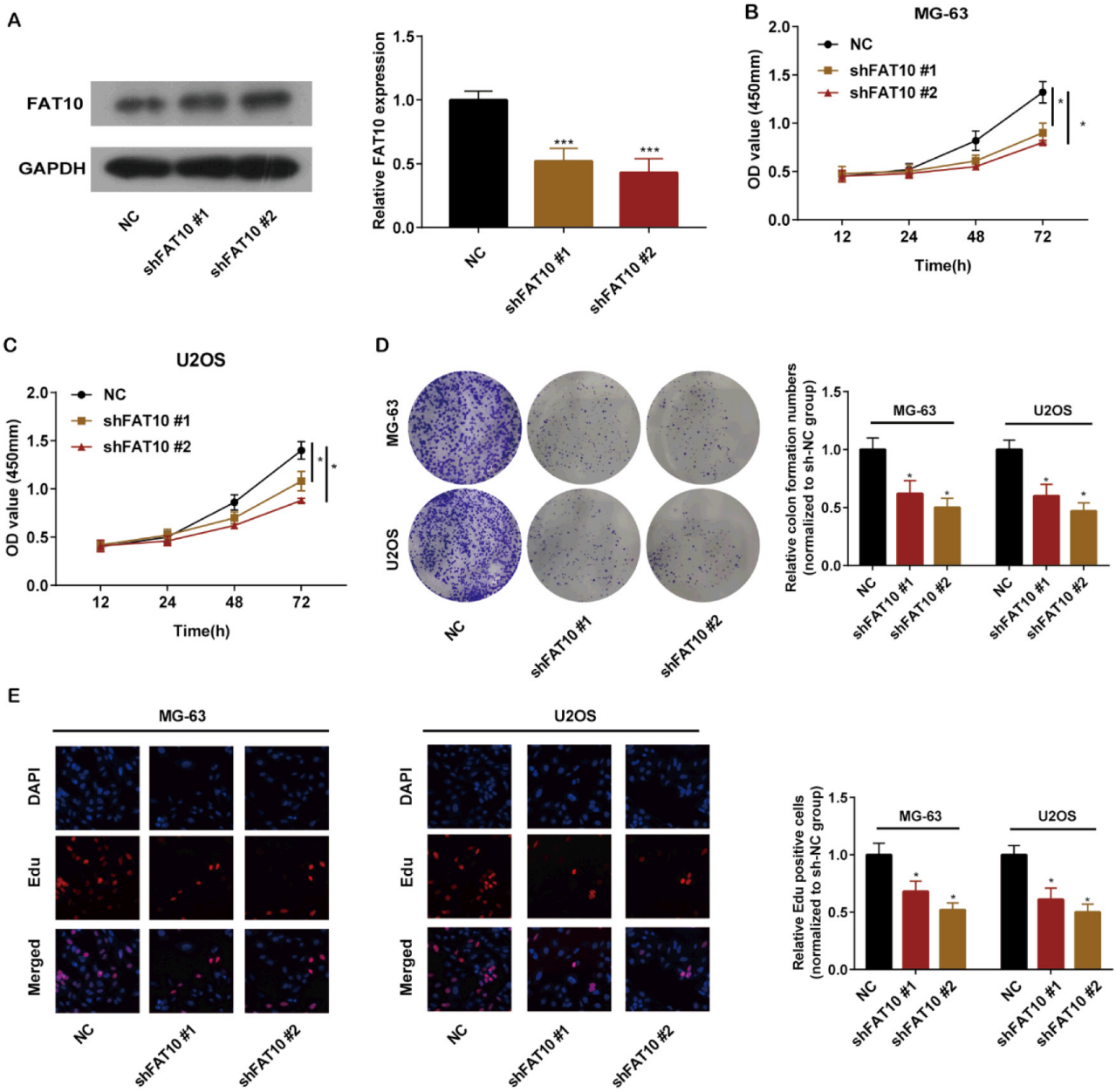

Figure 2. Intervention of FAT10 suppressed the proliferative capacity of OS. **A:** Protein level of FAT10 in MG-63 cells transfected with NC, shFAT10#1 or shFAT10#2 for 48 h detected by Western blot. **B,C:** Cell viability in MG-63 **(B)** and U2OS cells **(C)** transfected with NC, shFAT10#1 or shFAT10#2 for 48 h detected by CCK-8 assay. **D:** Colony formation in MG-63 and U2OS cells transfected with NC, shFAT10#1 or shFAT10#2 for 48 h. **E:** EdU-positive MG-63 and U2OS cells transfected with NC, shFAT10#1 or shFAT10#2 for 48 h detected by EdU assay (*p<0.05).

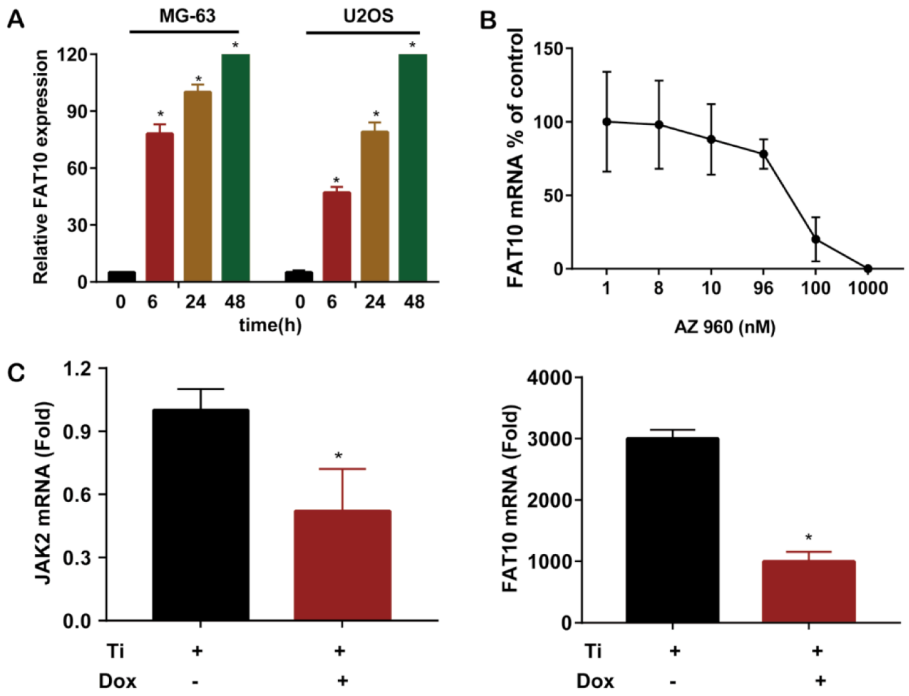

Figure 3. FAT10 was regulated by the JAK/STAT signaling pathway. **A:** The mRNA level of FAT10 in MG-63 cells (left) and U2OS cells (right) stimulated with IFN-γ and TNF-α for 0, 6, 24 and 48 h; **B:** The mRNA level of FAT10 in MG-63 cells induced with 1, 8, 10, 96, 100 and 1000 nM AZ960 for 24 h. **C:** The mRNA levels of JAK2 (left) and FAT10 (right) in MG-63 cells pretreated with Ti for 24 h, followed by 72-h treatment of Dox or not (*p<0.05).

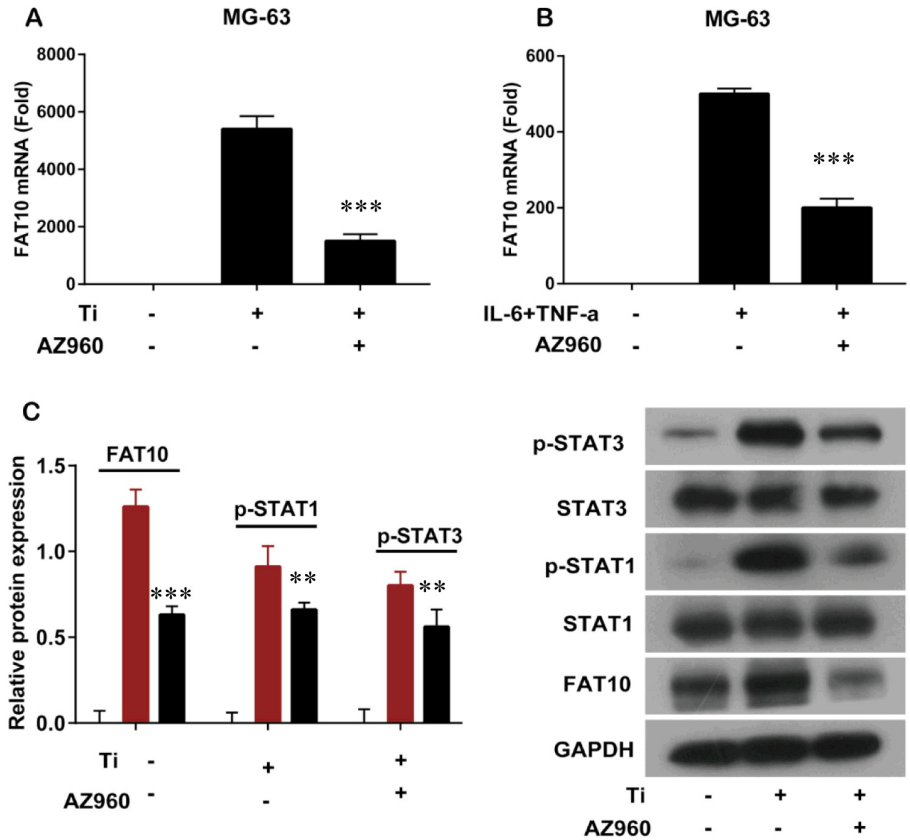

Figure 4. JAK2 was the mediator of FAT10 induced by IL-6 and TNF-α. **A:** The mRNA level of FAT10 in MG-63 cells pretreated with Ti for 24 h, followed by 24-h treatment of AZ960 or not. **B:** The mRNA level of FAT10 in MG-63 cells pretreated with IL-6 and TNF-α for 24 h, followed by 24-h treatment of AZ960 or not. **C:** Protein levels of p-STAT3, STAT3, p-STAT1, STAT1 and FAT10 in MG-63 cells pretreated with Ti for 24 h, followed by 24-h treatment of AZ960 or not (**p<0.01; ***p<0.001).

already known that STAT1 and STAT3 can be phosphorylated by JAK2. Our Western blot results identically revealed that protein levels of p-STAT1 and p-STAT3 were downregulated by the treatment of JAK2 inhibitor (Figure 4C). Therefore, JAK2 could regulate FAT10, and its transcription was induced *via* activating p-STAT1/3.

Discussion

OS is a highly malignant disease that usually affects adolescents [21]. The 5-year survival of OS remains low although therapeutic strategies have been advanced [22]. It is of great significance to clarify the pathogenesis of OS. FAT10 is reported highly expressed in multiple types of cancers like liver cancer, breast cancer and colorectal cancer [23-25]. Consistently, our results also showed that FAT10 was upregulated in OS specimens and cell lines, which was correlated to tumor size, WHO grade and distant metastasis of OS patients. A series of functional experiments proved that intervention of FAT10 inhibited viability, clonality and proliferative capacity of OS cells, indicating the vital role of FAT10 in the proliferative process of OS.

FAT10, also known as ubiquitin D, is a ubiquitin-like protein that was initially discovered in the HLA-F gene by Fan et al in 1996 [26]. FAT10 is closely related to cancer development *via* mediating functional behaviors of cancer cells [27]. It was previously reported that FAT10 is capable of elevating invasive and migratory rates of cells through the NF-κB-CXCR4/7 signaling pathway [28]. Through activating the Akt/GSK3b signaling pathway, FAT10 enhances the ability of liver cancer cells to invade [29]. Its potential function in OS, however, is unclear.

Pro-inflammatory factors can stimulate the expression of FAT10 [30]. During the course of inflammatory response, released cytokines (e.g. IL-6, IL-10 and IL-17) transport intracellular signals by STAT3, and activate the expression levels of genes relevant to cell cycle progression, cell survival and oxidative stress [31]. The binding of IL-6 with its receptors produces various effects, and the most studied one is the activation of the JAK/STAT signaling pathway. IL-6 receptors have a relationship with tyrosine kinases of the JAK family, including JAK1, JAK2 and TYK2 [32,33]. After activation and self-phosphorylation of JAKs, phosphorylated STAT is dimerized and translocated in the nuclei as a transcription factor. In our study, to further investigate the involvement of JAK in FAT10-induced OS development in a pro-inflammatory environment, a well-known JAK2 inhibitor, AZ960 was used to stimulate OS cells. The mRNA level of FAT10 was dose-dependently downregulated in AZ960-induced OS cells, confirming the important role of the JAK signaling pathway. JAK2 is a vital mediator in hematopoietic development [34]. The positive expression of FAT10 in immune cells suggested that the JAK/STAT signaling pathway may be functional in regulating FAT10. JAK2 is able to phosphorylate STAT1, STAT3 and STAT3. Here, Western blot analysis showed that AZ960 treatment downregulated FAT10, p-STAT1 and p-STAT3 in proinflammatory factors-induced OS cells, whilst total STAT1 and STAT3 were not influenced. Collectively, the effect of FAT10 on OS cells induced by proinflammatory stimulation was mediated by the JAK2/STAT signaling pathway.

Conclusions

FAT10 promotes OS cells to proliferate in a pro-inflammatory environment by activating the JAK2 signaling pathway. FAT10 can be utilized as novel therapeutic and diagnostic targets of OS.

Conflict of interests

The authors declare no conflict of interests.

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