Original Paper

Serine/Threonine Kinase 35, a Target Gene of STAT3, Regulates the Proliferation and Apoptosis of Osteosarcoma Cells

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Key Words

Stk35 • Osteosarcoma • STAT3 • Apoptosis

Abstract

Background/Aims: Serine/threonine kinase 35 (STK35) may be associated with Parkinson disease and human colorectal cancer, but there have been no reports on the expression levels or roles of STK35 in osteosarcoma. **Methods:** STK35 mRNA expression was determined in osteosarcoma and bone cyst tissues by real-time PCR. Cell proliferation and apoptosis were assessed by Cell Counting Kit-8 (CCK-8) assay and flow cytometry analysis, respectively. **Results:** STK35 was up-regulated in osteosarcoma tissues as indicated by analyzing publicly available expression data (GEO dataset E-MEXP-3628) and real-time PCR analysis on our own cohort. We subsequently investigated the effects of STK35 knockdown on two osteosarcoma cell lines, MG63 and U2OS. STK35 knockdown inhibited the growth of osteosarcoma cells *in vitro* and in xenograft tumors. Meanwhile, STK35 knockdown enhanced apoptosis. Expression of the active forms and the activity of two major executioner caspases, caspase 3 and caspase 7, were also increased in osteosarcoma cells with STK35 silenced. Additionally, Gene Set Enrichment Analysis (GSEA) identified that the JAK/STAT signaling pathway was positively correlated with STK35 expression. The mRNA expression of STK35 was repressed by STAT3 small interfering RNA (siRNA), but not by siRNA of STAT4, STAT5A or STAT6. A luciferase reporter assay further demonstrated that STAT3 transcriptionally regulated STK35 expression. A chromatin immunoprecipitation (ChIP) assay confirmed the direct recruitment of STAT3 to the STK35 promoter. The promotion effects of STAT3 knockdown on cell apoptosis were partially abolished by STK35 overexpression. Furthermore, STK35 mRNA expression was positively correlated with STAT3 mRNA expression in osteosarcoma tissues by Pearson correlation analysis. **Conclusions:** These results collectively reveal that STAT3 regulates the transcription of STK35 in osteosarcoma. STK35 may exert an oncogenic role in osteosarcoma.

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Introduction

Osteosarcoma is the most common primary malignancy of the bone, which particularly affects children and young adults with a 5-year survival rate of approximately 70% [1]. However, poor prognosis was observed in metastatic and recurrent osteosarcoma [2]. A better understanding of the underlying mechanism of the carcinogenesis of osteosarcoma is crucial for the diagnosis of and the treatment for osteosarcoma. A variety of signaling pathways, such as hedgehog [3], vascular endothelial growth factor (VEGF) [4, 5], platelet-derived growth factor (PDGF) [6], Wnt [7] and signal transducer and activator of transcription (STAT) pathways [8], have been demonstrated to mediate cell proliferation, apoptosis or invasion of osteosarcoma cells.

Serine/threonine kinase 35 (STK35) is a member of the serine/threonine protein kinase family [9]. A few studies have investigated the biological function of STK35. STK35 may regulate CDKN2A expression, G1- to S-phase transition and migration of endothelial cells [10]. Ectopic expression of STK35 enhanced caspase-independent cell death of cells undergoing oxidative stress [11]. In addition, studies also suggest that STK35 may be involved in several human diseases. By kinome-wide RNAi screening, STK35 silencing was found to decrease the infection of hepatocytes by *Plasmodium* sporozoites [12]. STK35 gene expression was altered in human colorectal cancer [13] and in a rodent model of Parkinson disease [14]. However, little is known about the expression and roles of STK35 in osteosarcoma.

In the current study, we found that STK35 expression was higher in osteosarcoma tissues than that in control tissues. STK35 knockdown inhibited proliferation and induced apoptosis in osteosarcoma cells. In addition, a transcriptional regulatory effect of STAT3 on STK35 expression was discovered. Our data suggest that STK35 acts as an oncogene in osteosarcoma tumorigenesis and may be a potential biomarker and therapeutic target for osteosarcoma.

Materials and Methods

Tissue specimens and cell lines

Tissue specimens, including 20 bone cysts and 40 osteosarcoma tissues, were obtained from Shanghai Tenth People's Hospital. These tissue specimens were snap-frozen in liquid nitrogen and stored at -80°C until use. This study was approved by the Ethics Committee of Shanghai Tenth People's Hospital, and all patients gave written informed consent.

The osteosarcoma cell lines used in the present study, such as U2OS, SOSP-9607 MG63, HOS and SaoS2, were obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China) and regularly grown in respective culture medium (RPMI or DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. All the cell lines were maintained in a humidified incubator with 5% carbon dioxide at 37°C.

RNA extraction and real-time PCR

Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Oligo (dT)₁₈-primed cDNA was synthesized with 2 µg DNase-treated RNA by using the cDNA First-Strand Synthesis kit

Table 1. Primer pairs used for real-time PCR

Gene	Primers (forward/reverse)
STK35	5'-AGACCTCGCTGAAAGGAGAAA-3' and 5'-CCGGGACAGGACATACTGATT-3'
STAT3	5'-CAGCAGCTTGACACACGGTA-3' and 5'-AAACACCAAAAGTGGCATGTGA-3'
STAT4	5'-TGTTGGCCCAATGGATTGAAA-3' and 5'-GAAACACGACCTAAGTGTTCAT-3'
STAT5A	5'-CGACGGGACCTTCTTGTG-3' and 5'-GTTCCGGGGAGTCAAACCTTC-3'
STAT6	5'-GCCAAAGCCCTAGTGCTGAA-3' and 5'-GACGAGGGTCTCAGGACTTC-3'
GAPDH	5'-CACCACCTCCTCCACCTTTG-3' and 5'-CCACCACCTGTGTGTAG-3'
STK35 promoter	5'-CGGTGTGGTCTTCACGCAGAT-3' and 5'-ATAGGCGCGAAATCCCGTG-3'
3'UTR	5'-TGGGTGATTTTAACTAGGTCGAT-3' and 5'-ATTGCCGGATCCAGCTGTC-3'

(Fermentas, Hanover, MD, USA). Quantitative real-time PCR was then conducted by using a SYBR Green PCR kit (Thermo Scientific, Rockford, IL, USA) on an ABI 7300 instrument (Applied Biosystems, Foster City, CA, USA). Melting curves were performed to verify the specific product amplification. Primers are listed in Table 1. All reactions were run in triplicate, and mRNA expression levels were normalized to GAPDH.

Immunoblotting analysis

Whole protein extracts were prepared in radioimmunoprecipitation assay buffer (Solarbio, Shanghai, China) with protease and phosphatase inhibitors. Protein concentration was estimated by a BCA protein kit (Thermo Scientific). Equal amounts of protein were resolved on 10% polyacrylamide gels via SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Millipore, Bedford, MA, USA). After blocking with 5% nonfat dry milk for 1 h at room temperature, the membranes were probed at 4°C overnight with primary antibodies followed by secondary antibodies at room temperature for 1 h. An enhanced chemiluminescence system (Millipore) was used for the detection of protein expression. The primary antibodies against STK35, active caspase 3 and active caspase 7 were purchased from Abcam (Cambridge, MA, USA), while antibodies against STAT3 and GAPDH were from Cell Signaling Technology (Danvers, MA, USA).

Lentiviral-mediated RNA interference and overexpression of STK35

Lentiviral plasmids (pLKO.1) containing short hairpin RNA (shRNA) directed to various regions of human *STK35* gene (RNAi-1, 5'-GCATTATCATCTGGGCAAT-3'; RNAi-2, 5'-AGAGGGCAATCAAGACAAC-3'; RNAi-3, 5'-GATATGTTAGCTGCTAACC-3') or containing normal control shRNA (NC, 5'-CCTAAGGTTAAGTCGCCCTCG-3'), as well as a lentiviral plasmid (pLVX-puro) containing the full length of human *STK35* cDNA sequence, were constructed. Lentiviral transfection was carried out in HEK293T cells by using lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions. At 48 hours after transfection, the viral supernatant was collected and added to osteosarcoma cells in the presence of polybrene (10 mg/ml; Sigma, St. Louis, MO, USA). *STK35* expression was evaluated after 48 h of transduction.

RNA interference of STATs

The small interfering RNAs (siRNAs) against STAT3 (5'-GGCCAGCAAAGAAUCACAU-3'), STAT4 (5'-GAGUCCACAACAAUUGAA-3'), STAT5A (5'-GCGCUUUGAGUCAGAA-3') and STAT6 (5'-CAGUCCGCCACUUGCCAA-3'), as well as a scramble normal control siRNA (NC) were synthesized by GenePharma (Shanghai, China). MG63 and HOS cells were transfected with the siRNAs by using lipofectamine 2000, and the expressions of STATs and *STK35* were assessed after 48 h of incubation.

Luciferase reporter assay

The full length *STK35* promoter was inserted into a pGL3 vector (Promega, Madison, WI). An expression plasmid (pCDNA3.1) containing the full length of human *STAT3* cDNA sequence was constructed. MG63 and HOS cells were co-transfected with the pGL3-*STK35* promoter and *STAT3* siRNA or control siRNA and/or with a *STAT3*-expressing plasmid or vector. Luciferase activity was measured using a luciferase assay kit (Promega) per the manufacturer's protocols.

Chromatin immunoprecipitation (ChIP) assay

ChIP was performed as previously described [15]. In brief, MG63 and HOS cells (1×10^7 cells) were treated with 1% formaldehyde at room temperature for 10 min and lysed in sodium dodecyl sulfate (SDS)-containing lysis buffer. After sonification, the supernatant was collected following centrifugation at 8,000 $\times$ g for 10 min and incubated overnight with *STAT3* antibody or control IgG. The immune complex was then captured by protein A/G beads. The purified DNA from the immune complex was subjected to SYBR green real-time PCR. The 3' untranslated regions (UTRs) were used as negative controls. Primers are listed in Table 1.

Cell proliferation assay

Cell proliferation was determined using a Cell Counting Kit-8 (CCK-8) assay. MG63 and HOS cells were plated into 96-well plates (3×10^3 cells per well) and cultured overnight. The cells were then infected with *STK35* shRNA virus or control shRNA virus. After incubation for 0, 24, 48 and 72 h, CCK-8 reagent (SAB

Biotech, College Park, MD, USA) was added and incubated for 1 h in standard culture conditions. Absorbance at 450 nm was measured using a microplate reader.

Cell apoptosis assay

At 48 h after viral infection, MG63 and HOS cells were collected and stained with Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (Beyotime) according to the manufacturer's instructions. Subsequently, cells were analyzed in a flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Caspase 3/7 activity assay

At 48 h after viral infection, caspase 3/7 activity of MG63 and HOS cells was measured using the Caspase 3/7 Activity kit (AAT Bioquest, Sunnyvale, CA, USA) as per the manufacturer's protocol. Briefly, cells were incubated with caspase 3/7 assay solution, which contained caspase substrate (Z-DEVD-R110), at room temperature for 1 h in the dark. Fluorescence intensity was then measured at 490 nm excitation and 525 nm emission. The results are expressed as a percentage of the mean of the control group (set at 100%).

Xenografts in nude mice

Animal experiments were approved by the Animal Experimentation Ethics Committee of Shanghai Tenth People's Hospital. Four- to six-week-old male nude mice (n=6 per group) purchased from Shanghai Experimental Animal Center (Shanghai, China) were maintained in specific pathogen free (SPF) conditions with free access to sterile water and chow. MG63 cells infected with STK35 shRNA (RNAi-1) or NC were subcutaneously injected into the flank of nude mice (5×10^5 cells per mouse). The length, width and height of xenografts were measured every three days using calipers and tumor volume was calculated using the formula, volume = length $\times$ width $\times$ height $\times$ 0.5. At 33 days after cell injection, the xenografts were collected and weighed. STK35 mRNA and protein expression in xenografts were detected by real-time PCR and immunoblotting, respectively.

Online dataset analysis

STAT3 mRNA levels in PubMed GEO dataset (E-MEXP-3628, 14 osteosarcoma tissues and 4 normal bone tissues) [16] were analyzed.

To investigate STK35-associated pathways in osteosarcoma, the osteosarcoma expression data from E-MEXP-3628 were divided into STK35 high and STK35 low groups, and Gene Set Enrichment Analysis (GSEA) was performed as previously described [17].

Statistical analysis

All statistical analyses were carried out using GraphPad Prism statistical software (GraphPad Software, San Diego, CA, USA). Student's *t*-test was used to compare differences between two groups. One-way analysis of variance (ANOVA) with a post-test was used to compare three or more groups. P values less than 0.05 were considered to indicate statistical significance.

Results

STK35 overexpression in osteosarcoma tissues

By analyzing publicly available expression profiles obtained from 14 osteosarcoma tissues and 4 normal bone tissues (GEO dataset, E-MEXP-3628), we found that STK35 was significantly overexpressed in osteosarcoma tissues com-

Fig. 1. STK35 expression in osteosarcoma tissues. (A) STK35 expression in the E-MEXP-3628 dataset, which included 14 osteosarcoma tissues and 4 normal bone tissues. (B) STK35 expression measured with real-time PCR in osteosarcoma tissues (n=40) as compared to bone cysts (n=20). GAPDH served as an internal control. Student's *t*-test P value is shown. *** $P < 0.001$, **** $P < 0.0001$.

pared to normal human bone tissues ($P < 0.0001$, Fig. 1A). Moreover, real-time PCR analysis showed that STK35 was overexpressed in 40 osteosarcoma samples compared to 20 bone tissues ($P < 0.001$, Fig. 1B) <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4745698/figure/F1/>. These findings suggest that STK35 expression is increased in osteosarcoma.

Knockdown of STK35 expression by shRNA

To facilitate the investigation of the pathological role of STK35 expression in osteosarcoma progression, we knocked down its expression in osteosarcoma cell lines. Real-time PCR and immunoblotting analyses indicated that MG63 and HOS cells showed relatively higher levels of STK35 expression than the other three cell lines (U2OS, SOSP-9607 and SaoS2; Fig. 2A). Thus, these two cell lines were chosen for knockdown studies.

Lentivirus plasmid vectors containing STK35 shRNAs (RNAi-1, RNAi-2 and RNAi-3) or control shRNA (NC) inserts were constructed, and lentiviruses were then produced to transduce MG63 and HOS cells. As shown in Fig. 2B and 2C, NC had no effects on STK35 expression compared to that in wild-type (WT) cells. All STK35 shRNAs significantly down-regulated the expression of STK35 ($P < 0.001$) compared with that in NC and WT cells. Among the three shRNAs, RNAi-1 was the most efficient one with a knockdown ratio of $>70\%$ in both cell lines. Thus, RNAi-1 was used for knockdown studies.

Effects of STK35 knockdown on osteosarcoma cell proliferation

To elucidate the roles of STK35 in osteosarcoma cell proliferation, a CCK-8 assay was performed every 24 h in MG63 and HOS cells as described above. The results demonstrated that cells with STK35 knockdown (RNAi-1) exhibited a significant reduction in proliferation ability compared with that in the NC and WT cells ($P < 0.01$, Fig. 3A and 3B).

Fig. 2. Knockdown of STK35 expression by shRNA. (A) mRNA (left panel) and protein (right panel) levels of STK35 measured with real-time PCR and immunoblotting, respectively, in osteosarcoma cell lines. GAPDH was an internal control. Representative blots from three repeats are shown. (B, C) MG63 (B) and HOS (C) cells were infected with STK35 shRNAs (RNAi-1, RNAi-2 and RNAi-3) or control shRNA (NC) virus. Wild-type (WT) cells without any treatment served as a negative control. STK35 mRNA and protein levels were assessed at 48 h after viral infection. *** $P < 0.001$.

Fig. 3. Effects of STK35 knockdown on osteosarcoma cell proliferation. (A, B) MG63 (A) and HOS (B) cells were infected with STK35 shRNA (RNAi-1) or control shRNA (NC) virus. Cell proliferation was evaluated at 0, 24, 48 and 72 h after viral infection. WT cells without any treatment served as a negative control. ** $P < 0.01$, *** $P < 0.001$. (C-E) STK35 knockdown inhibited MG63 cell growth in vivo. Tumor volumes of RNAi-1 group and NC group (C) were calculated every three days ($n=6$ /group). At 33 days after cell inoculation, tumors were resected and weighed (D), and STK35 mRNA and protein levels in tumors were measured (E). Representative blots from one pair of samples are shown.

Given the *in vitro* findings, mouse xenograft studies were conducted to further assess the effect of STK35 on tumor growth *in vivo*. Two groups of stable cells (NC group, MG63 cells stably transduced with control shRNA; and RNAi-1 group, MG63 cells stably transduced with STK35 shRNA) were injected into the flanks of nude mice. The growth curves of xenograft tumors demonstrated that the RNAi-1 group produced tumors much more slowly than the NC group (Fig. 3C). Thirty-three days after inoculation, the xenograft tumors were recovered. The tumor weight of the RNAi-1 group (0.198 ± 0.0250 g) was lighter than that of the NC group (0.7165 ± 0.0370 g; $P < 0.001$, Fig. 3D). Meanwhile, mRNA and protein levels of STK35 in the xenograft tumors were also tested. The results showed that STK35 expression was significantly lower in the RNAi-1 group than in the NC group (Fig. 3E). These data demonstrate that STK35 knockdown inhibits osteosarcoma cell growth *in vivo*.

Enhanced apoptosis by STK35 knockdown

We next investigated whether the impaired proliferation of osteosarcoma cells with STK35 knockdown was due to an increase in apoptosis. By flow cytometry analysis on cells with Annexin V/PI-double labeling, we found that STK35 shRNA treatment in both MG63 and HOS cells significantly enhanced apoptosis compared with that in the NC and WT groups ($P < 0.001$). The cells in the NC and WT groups had similar apoptosis rates (Fig. 4A and 4B).

Moreover, the protein levels of active caspase 3 and active caspase 7 were evaluated by immunoblotting. STK35 shRNA treatment (RNAi-1) caused a decline in STK35 expression and an increase in the expression of active caspase 3 and active caspase 7 (Fig. 4C). The results of the caspase 3/7 activity assay (Fig. 4D) were consistent with the immunoblotting results. All of these data indicated that STK35 knockdown promoted apoptotic cell death in osteosarcoma cells.

Fig. 4. Enhanced apoptosis by STK35 knockdown. (A, B) Annexin V/propidium iodide (PI) staining was performed in STK35 knockdown (RNAi-1) and control (WT and NC) MG63 (A) and HOS (B) cells, and cell apoptosis was analyzed by flow cytometry. (C) Protein expression levels of STK35, active caspase 3 and active caspase 7 were evaluated. Representative blots from three repeats are shown. (D) Caspase 3/7 activity was measured using the Caspase 3/7 Activity kit. The results are expressed as a percentage of the mean of the WT group (set at 100%). *** $P < 0.001$.

STK35 is regulated by STAT3 in osteosarcoma

To investigate STK35-associated pathways in osteosarcoma, GSEA was conducted on the osteosarcoma samples (E-MEXP-3628) with higher STK35 expression versus lower STK35 expression. The Kyoto Encyclopedia of Genes and Genomes (KEGG) JAK/STAT signaling pathway was identified as positively correlated with STK35 expression (Fig. 5A and Table 2).

STAT transcription factors are known to regulate cell apoptosis, proliferation, differentiation and migration via the downstream target genes [18]. We hypothesized that STK35 expression was regulated by STAT family proteins in osteosarcoma and tested it by evaluating STK35 expression of MG63 and HOS cells after knocking down the expression of STAT family proteins (STAT3, STAT4, STAT5A and STAT6). Successful knockdown of the STAT family proteins was evident by real-time PCR analysis (Fig. 5B). STK35 mRNA expression was suppressed by STAT3 silencing ($P < 0.001$) but not by the depletion of other STAT members (Fig. 5C). Immunoblotting analysis showed that STAT3 knockdown also decreased the protein levels of STK35 (Fig. 5D).

Further, we examined whether STAT3 can impact on the activity of the STK35 promoter. As illustrated in Fig. 5E, the relative luciferase activity was markedly enhanced when the STK35 promoter was co-transfected with the STAT3 overexpression plasmid, while relative luciferase activity was notably reduced when the STK35 promoter was co-transfected with STAT3 siRNA (Fig. 5E). These data demonstrated that STAT3 was responsible for the promoter activity and transcription of STK35.

Using PROMO (<http://www.lsi.upc.es/cgi-bin/user/>

Table 2. GSEA in osteosarcoma tissues (E-MEXP-3628). *P<0.01

NAME	SIZE	ES	NES	NOM p-value
KEGG_JAK_STAT_SIGNALING_PATHWAY	150	0.3235	1.3659	0.0291*
PID_WNT_SIGNALING_PATHWAY	27	0.3459	1.1080	0.3233
KEGG_T_CELL_RECEPTOR_SIGNALING_PATHWAY	107	0.2435	0.9901	0.4965
KEGG_MAPK_SIGNALING_PATHWAY	257	0.2192	0.9738	0.5636
KEGG_PPAR_SIGNALING_PATHWAY	67	0.2525	0.9559	0.5469
KEGG_CHEMOKINE_SIGNALING_PATHWAY	179	0.2136	0.9129	0.6928
KEGG_VEGF_SIGNALING_PATHWAY	70	0.2321	0.8824	0.6667
BIOCARTA_PDGF_PATHWAY	32	0.2352	0.7722	0.8151
KEGG_HEDGEHOG_SIGNALING_PATHWAY	54	0.2078	0.7497	0.8804
BIOCARTA_AT1R_PATHWAY	30	0.2064	0.6781	0.8946
KEGG_ERBB_SIGNALING_PATHWAY	87	0.1622	0.6394	0.9838
PID_RHOA_REG_PATHWAY	44	0.1772	0.6227	0.9678
BIOCARTA_IL10_PATHWAY	17	0.2177	0.6168	0.9256
KEGG_TOLL LIKE RECEPTOR SIGNALING_PATHWAY	99	0.1490	0.6023	0.9943

Fig. 5. STK35 is regulated by STAT3 in osteosarcoma. (A) GSEA analysis identified that the KEGG JAK/STAT signaling pathway was positively correlated with STK35 expression. NES, normalized enrichment score. (B, C) STAT3, STAT4, STAT5A and STAT6 were knocked down in MG63 and HOS cells by specific siRNA transfection. The mRNA levels of STATs (B) and STK35 (C) were then measured by real-time PCR. (D) The protein levels of STAT3 and STK35 after STAT3 knockdown were assessed by immunoblotting analysis. Representative blots from three repeats are shown. (E) The STK35 promoter was co-transfected into MG63 and HOS cells with a STAT3 overexpression plasmid (or control vector) or STAT3 siRNA (or control siRNA [NC]), and then luciferase reporter assays were performed to evaluate the activity of the STK35 promoter. (F) The ChIP assay demonstrated that STAT3 bound to the STK35 promoter. Top: Schematic diagram of primers for ChIP analysis. 3'UTR served as a negative control Bottom: ChIP assays were carried out in MG63 cells. BS: binding site. *** P<0.001.

Fig. 6. Association of STK35 and STAT3 in osteosarcoma cell apoptosis. SaoS2 cells were treated with an STK35 overexpression virus (or control vector) and STAT3 siRNA (or control siRNA [NC]). (A) Cell apoptosis was measured. (B) Protein expressions of STAT3, STK35, active caspase 3 and active caspase 7 were evaluated. Representative blots from three repeats are shown. (C) Caspase 3/7 activity was measured, and the results are expressed as a percentage of the mean of the WT group (set at 100%). *** $P < 0.001$.

Fig. 7. Correlation analyses in osteosarcoma tissues. (A) STAT3 mRNA expression measured with real-time PCR in osteosarcoma tissues (n=40) as compared to bone cysts (n=20). *** $P < 0.001$. (B) Pearson correlation scatter plots in osteosarcoma tissues (n=40).

alggen/promo/promo/dynmat.cgi), a promoter analysis algorithm, a STAT3 binding site was predicted in the STK35 promoter (-153 to -142). To confirm the direct binding of STAT3 to the STK35 promoter, a ChIP assay was performed in MG63 cells. As shown in Fig. 5F, STAT3 directly bound to the STK35 promoter (-230 to -132), but not to the 3'UTR.

Association of STK35 and STAT3 in osteosarcoma cell apoptosis

To test the association of STK35 and STAT3 in osteosarcoma cell apoptosis, we overexpressed STK35 in osteosarcoma cells with STAT3 silenced. Here, SaoS2 cells were chosen because of their relatively lower expression levels of STK35. Results of the flow cytometry analysis showed that STAT3 knockdown remarkably promoted cell apoptosis, which was partially abrogated by STK35 overexpression (Fig. 6A). The expression of STK35 and STAT3 was verified by immunoblotting analyses (Fig. 6B). The changes in the active forms of caspase 3 and caspase 7 (Fig. 6B and 6C) were consistent with the results of the flow cytometry analysis. These data indicated that STK35 was a downstream effector of STAT3 in the regulation of osteosarcoma cell apoptosis.

Correlation analyses in osteosarcoma tissues

Real-time PCR analysis was performed to evaluate the mRNA expression of STAT3 in the samples and is shown in Fig. 1B. STAT3 was also significantly overexpressed in

osteosarcoma tissues compared to bone cysts ($P < 0.0001$, Fig. 7A). Pearson correlation analysis demonstrated a positive correlation between mRNA expression of STK35 and STAT3 in osteosarcoma tissues (Fig. 7B). These data indicate the regulation effects of STAT3 on STK35 expression in osteosarcoma tissues.

Discussion

A previous *in situ* hybridization study reported that STK35 gene expression is up-regulated in human colorectal cancer [13]. However, few investigations have been carried out to clarify the functions of STK35 in human cancers. In the current study, we first demonstrated the increased expression of STK35 in osteosarcoma tissues compared to that in control tissues. We provided evidence for the regulatory effects of STK35 on proliferation and apoptosis of osteosarcoma cells. Further, we proposed that STAT3 transcriptionally regulated STK35 expression.

First, STK35 was significantly overexpressed in osteosarcoma tissues compared to expression levels in normal human bone tissues and bone cysts by analyzing the expression profiles of the PubMed GEO dataset and real-time PCR results on samples obtained from our hospital, respectively (Fig. 1). Our findings indicated a possible pathological role of STK35 expression in osteosarcoma progression. Further, we explored the roles of STK35 on the proliferation of osteosarcoma cells by knocking down its expression. It has been reported that STK35 could regulate cell cycle transition of endothelial cells [10]. Here, an *in vitro* CCK-8 assay and *in vivo* xenograft experiments demonstrated that down-regulation of STK35 expression remarkably suppressed the proliferation of osteosarcoma cell lines (Fig. 3). Additionally, flow cytometry analysis demonstrated increased apoptosis of osteosarcoma cells with STK35 knockdown (Fig. 4), which may possibly lead to decreased proliferation. A previous study showed that ectopic expression of STK35 could enhance caspase-independent cell death under oxidative stress [11]. Here, STK35 knockdown significantly increased the activity of caspase 3 and caspase 7, two major executioner caspases [19] (Fig. 4C and 4D). These findings suggest that STK35 may play a key role in both caspase-independent cell death and caspase-induced cell apoptosis.

It has been reported that Importin $\alpha 2$ can bind to the promoter region of STK35 and induce its transcription [11]. The present report tried to investigate the upstream mediator of STK35 expression in osteosarcoma. First, we revealed that the JAK/STAT signaling pathway was positively correlated with STK35 expression by GSEA on a public dataset (Fig. 5A). STATs play key roles in numerous biological activities such as cell proliferation, apoptosis and differentiation via their target genes [18, 20, 21]. Thus, we hypothesized that STK35 expression was regulated by one or more members of the STAT family. Next, we demonstrated that the mRNA expression of STK35 was repressed by STAT3 siRNA but not by siRNA of STAT4, STAT5A or STAT6 (Fig. 5B-5D). A luciferase reporter assay further confirmed that STAT3 transcriptionally regulated STK35 expression (Fig. 5E). Further, consistent with the previous research [8], we found that inhibition of STAT3 induced the apoptosis of osteosarcoma cell lines. More importantly, the promotion effects of STAT3 knockdown on cell apoptosis were partially abrogated by STK35 overexpression (Fig. 6). Finally, STAT3 is known to be overexpressed in osteosarcoma tissues [8]. We demonstrated that STK35 mRNA expression was positively correlated with STAT3 mRNA expression in osteosarcoma tissues by Pearson correlation analysis (Fig. 7). Overall, these results infer that STAT3 regulates the transcription of STK35 in osteosarcoma.

Conclusion

In summary, our results demonstrate that STK35 is overexpressed in osteosarcoma tissues. Knockdown of STK35 expression significantly inhibits the proliferation of osteosarcoma cells, inducing apoptosis in these cells. STAT3 is a possible transcription factor that targets STK35 in osteosarcoma.

Disclosure Statement

The authors declare that they have no competing interests.

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