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Original Article

Hippo-YAP signaling pathway is associated with the prognosis in children with osteosarcoma

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Abstract: Background: Osteosarcoma is the most common bone malignancy in children and adolescents. In this study, we aimed to explore the association of Yap1 expression with the clinical characteristics and prognosis of patients with pediatric osteosarcoma. Methods: the expression of Yap1 in human osteosarcoma cell lines and tissues were detected in this study. Reverse transcriptase-PCR (RT-PCR), immunohistochemical staining and western blotting were used to determine the expression levels of Yap1 in 48 human OS samples. The association of Yap1 expression with the prognosis of patients with OS were analyzed by survival curves. Results: We found that LATS1, p-LATS1, Yap1 and p-Yap1 were highly expressed in four human osteosarcoma cells lines (HOS, Saos-2, U2OS, and MG-63). Further analysis showed that Yap1 expression was associated with the variables of tumor size ($P<0.05$), the presence of metastasis ($P<0.05$) and the response to pre-operative chemotherapy ($P<0.05$). patients with high Yap1 expression had significantly shorter survival times than those with low Yap1 expression. Over-expression of Yap1 was an independent prognostic factor of unfavorable survival in patients with osteosarcoma after multivariable adjustment. Conclusion: The expression of Yap1 was significantly higher in osteosarcoma tissues than non-tumorous tissues. Overexpression of Yap1 was an independently risk factor associated with the prognosis of patients with pediatric osteosarcoma.

Keywords: Hippo-YAP signaling, Yap1, osteosarcoma

Introduction

Osteosarcoma is a malignant tumor that cells occur in skeleton and affiliates, which has been reported to present aberrant growth and migration in osseous tissues [1, 2]. Osteosarcoma is the most common bone malignancy in children and adolescents and may lead to the possibility of other malignancy [3]. Previous studies have assessed clinical behavior, response to treatments, and factors affecting survival in maxillofacial osteosarcoma treated at a tertiary referral center [4, 5]. In recent years, new strategies have been proposed and suggested to improve the overall survival for patients with osteosarcoma [6, 7]. Major advances have been proposed for the treatment of osteosarcoma with the discovery of several chemotherapeutic, immunologic agents [8]. However, the overall survival remains with little improvement since the introduction of neoadjuvant chemotherapy, radiotherapy and surgery.

The Hippo/YAP pathway was originally found in the Drosophila [9, 10], which was highly conserved kinase cascade and was frequently associated with cellular proliferation, differentiation and survival, In the mammalian Hippo pathway, the central components of this pathway comprise a regulatory serine-threonine kinase module and a transcriptional module. Mammalian Ste20-like kinases (MST1/2) and large tumor suppressor kinases (LATS1/2) as kinase module regulate transcriptional coactivators, Yes-associated protein (YAP) and Transcriptional coactivator with a PDZ-binding motif (TAZ) [11, 12]. In humans, MST1/2 combines with Salvador family WW domain-containing protein 1 (SAV1) to form an activated complex that initiates LATS1/2 phosphorylation

Table 1. Clinicopathological features of patients with pediatric osteosarcoma

Variables	Number	Percent (%)
Gender		
Female	20	41.6
Male	28	58.4
Age in years		
≤13	30	63.5
>13	18	37.5
Anatomic location		
Tibia/femur	36	75
Eleswherre	12	25
Pathological facture		
Present	11	22.9
Absent	37	77.1
Tumour characteristics		
Tumor size		
< or =5 cm	21	43.8
>5 cm	27	56.2
Tumour distant Metastasis		
Yes	15	31.3
No	33	68.7
Clinical Stage		
I+IIA	17	35.4
IIB+III	31	64.6
Subtype of osteosarcoma		
Conventional	22	45.8
Non-conventional	26	54.2
Response to pre-operative chemotherapy		
Good	27	56.2
Poor	21	43.8
Expression of Yap1 levels		
High	18	37.5
Low	30	63.5

[13-15]. Once activated, LATS1/2 further promotes the signaling cascade by phosphorylating YAP at Ser127 or TAZ at Ser89, then activate expression of target genes regulating cell proliferation, differentiation, and apoptosis [16-18]. Transcription cofactor YAP does not contain its own DNA-binding motifs and initiates transcription by interacting with the DNA-binding transcription factors TEA domain (TEAD) family members. Hosphorylated YAP then binds to 14-3-3 protein and remains in the cytoplasm for degradation [19, 20]. Dephosphorylated YAP translocates into the nucleus and binds to TEAD1-4, which activates downstream genes to support proliferation and inhibit apoptosis [21, 22]. In addition, the control of TEAD activity by YAP and TAZ has been associated with increased cell motility [23, 24].

To explore the role of Hippo-YAP signaling pathway in pediatric osteosarcomas, we investigated the expression of Yap1 and p-Yap1, LATS1 and p-LATS1 in 48 patients with osteosarcomas. The expression of Yap1 was associaed with clinical parameters such as chemotherapy response, progression-free survival (PFS) and overall survival (OS).

Patients and materials

Patients, osteosarcoma specimen

48 children (age range: 4-20 years, median 13 years) with osteosarcomas tissues and corresponding noncancerous bone tissue samples from the same specimens were collected from Department of Surgery, Baoji Hospital affiliated to Xi'an Medical University between July 1, 2003 to May 15 2016. All patients were treated with preoperative chemotherapy lasting for 4 months, using either the combination of an anthracycline (doxorubicin) and high-dose methotrexate or the combination of etoposide, ifosfamide, and high-dose methotrexate. This study was approved by the Institutional Review Board of Xi'an Medical University. All patients were given written informed consent to participate. The data did not contain any information that could identify the patients. Patients were excluded from this study if they had a history of other solid tumors, or died from severe postoperative complications. The clinicopathological information of all patients was shown in **Table 1**.

Cell culture

Human osteosarcoma cell lines (HOS, Saos-2, U2OS, and MG-63) and normal osteoblast cells (NHOst) were obtained from the Chinese Cell Bank of the Chinese Academy of Sciences

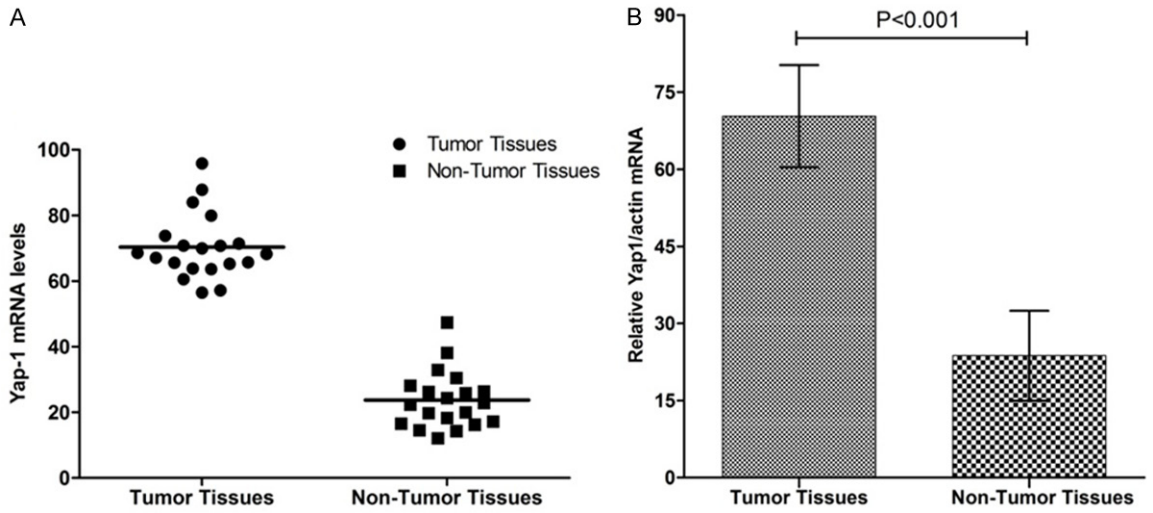

Figure 1. A, B: Upregulation of Yap1 mRNA levels in the OS tissues when compared with adjacent nontumorous tissues.

(Beijing, China). All cells and were cultured in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL of penicillin, and 100 μg/mL of streptomycin. They were all placed in a humidified atmosphere containing 5% CO_2 at 37°C.

Immunohistochemistry and evaluation of immunostaining

Immunohistochemical staining was performed with the Dako Envision Plus System (Dako, Carpinteria, CA) according to the manufacturer's instructions. For Yap1 staining, the Catalyzed Signal Amplification System (CSA II; DAKO) was employed according to the manufacturer's instructions using 3,3-diaminobenzidine as chromogen. Yap1 antibodies (Cell Signaling Technology) were used at a dilution of 1:100. The tissues were evaluated as positive for Yap1 staining when there were more than 10% of tumor cells demonstrating cytoplasmic and/or nucleus immune-reaction deposits. Yap1-positive cell numbers and staining intensity were blindly evaluated and further confirmed by two independent pathologists, and the final results regarding Yap1 expression level were obtained according to a previously validated scoring method [25].

RNA extraction and real-time reverse transcriptase-PCR

Total RNA of tissue samples were isolated using TRIzol (Life Technologies, Inc., Rockville, MD) according to the manufacturer's instructions. cDNA was generated from 1 μg of each RNA sample and a reverse transcribed using a transcription kit (Takara, Kyoto, Japan). Real-time quantitative reverse transcriptase-PCR (RT-PCR) was done in the 7300 Real Time PCR System (Applied Biosystems) Sense and antisense primers were synthesized based on the report by for human Yap-1 mRNA [26]. β-Actin served as an endogenous control. The Yap-1 mRNA levels were normalized to those of β-Actin mRNA. All measurements were repeated three times.

Western blotting analysis

Fresh surgical specimens were snap frozen in liquid nitrogen and stored in deep freezer. The normal tissues and the tumor were lysed in T-PER Tissue Protein Extraction Reagent (Pierce, Rockford, IL) containing proteinase inhibitors (CalBiochem, San Diego, CA). Protein concentrations were measured by the BCA Protein Assay Kit according to the manual instruction (Beyotime, Shanghai, China). Fifty micrograms denatured protein samples was separated by polyacrylamide gel electrophoresis (PAGE), transferred into PVDF membrane, and then incubated overnight with the primary antibodies: anti-Yap1 and anti-p-Yap1, anti-LATS1 and anti-p-LATS1 Cell Signaling Technology Inc., Beverly, MA), we also used β-actin as a loading control.

Statistical analysis

Statistical Package for the Social Sciences (SPSS) version 16.0 program was used for sta-

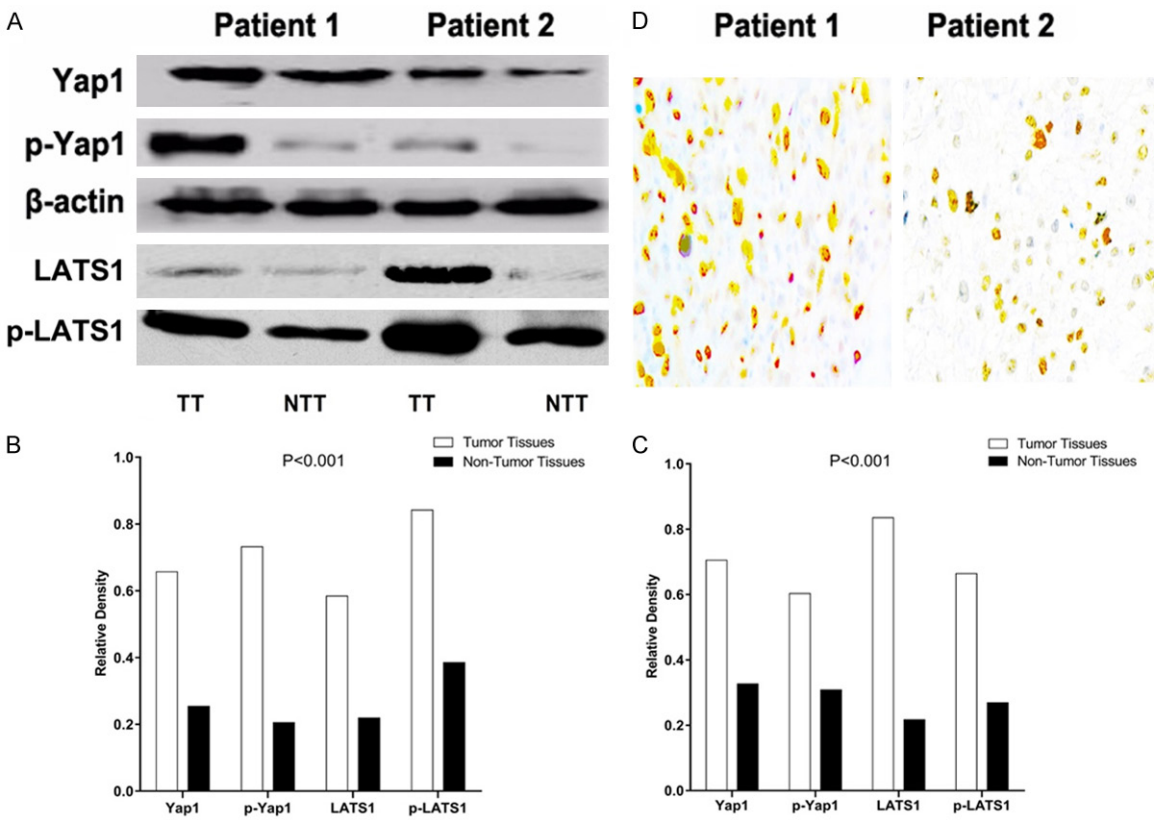

Figure 2. Upregulation of Yap1 and p-Yap1 protein levels in the OS tissues when compared with adjacent nontumorous tissues. A-C: Upregulation of Yap1, p-Yap1 LATS1 and p-LATS1 in protein levels in patient 1 and patients 2; D: Positive Yap1 IHC staining were observed patient 1 and patient 2.

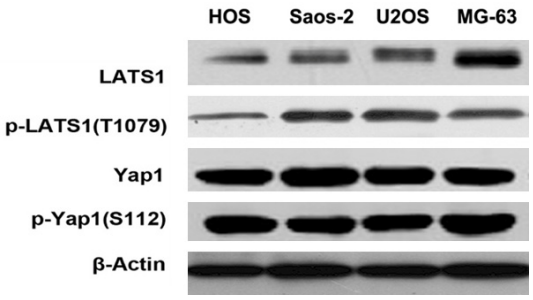

Figure 3. The expression of LATS1, p-LATS1, Yap1 and p-Yap1 were examined in four human osteosarcoma cells lines (HOS, Saos-2, U2OS, and MG-63) using Western blot.

tistical analysis. In the evaluation of study data, in addition to descriptive statistical methods (mean, standard deviation, median, frequency, rate, minimum, maximum), Student-t test was used for comparing variables showing normal distribution and Mann Whitney U test for comparing variables not showing normal distribution within qualitative data. Pearson's Chi-Square test, Fisher's Exact Test and Yates Continuity Correction Test were used for comparing qualitative data. Kaplan Meier survival analysis and log-rank were used for evaluating survival. Statistical significance was evaluated at the level of $P<0.05$.

Results

Expression of Yap1 and other factors in osteosarcoma tissues and cell lines

To take an insight into the role of Yap1 in OS, we evaluated Yap1 expression in 30 pairs of freshly isolated OS tissues and adjacent non-tumorous tissues by quantitative reverse transcription polymerase chain reaction (qRT-PCR) and Western blot. We found that significant upregulation of Yap1 were detected in mRNA levels a in the OS tissues when compared with adjacent nontumorous tissues ($P<0.01$, **Figures 1A**, **1B**, **2A**, **2C**). The expression of LATS1, p-LATS1 Yap1 and p-Yap1 were highly expressed in shown in OS tissues when compared with adja-

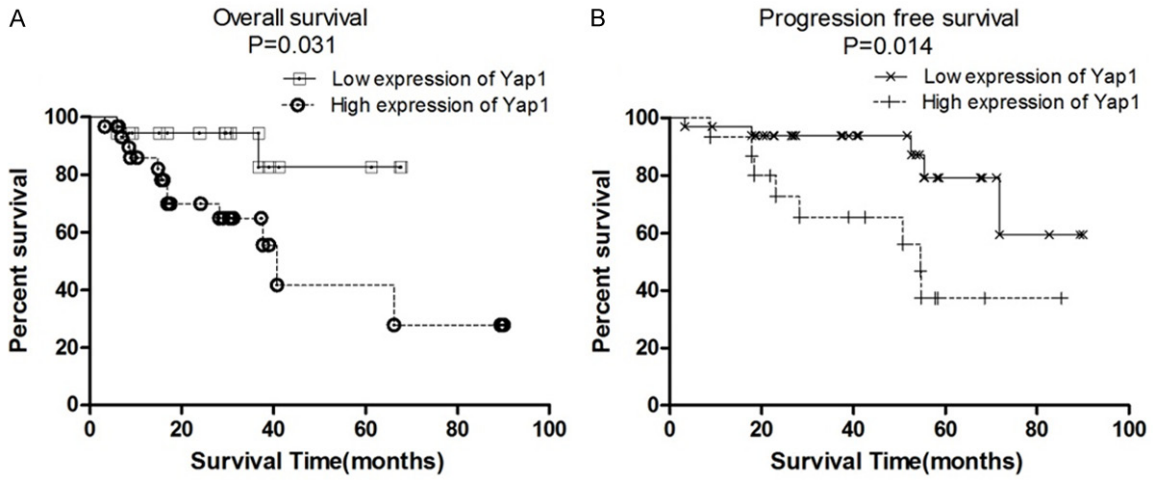

Figure 4. Kaplan-Meier analysis of OS (A) of patients with PFS (B) after stratification to Yap1 levels.

Table 2. Multivariable Cox proportional hazard regression analysis of patients' demographic and clinical characteristics and survival

Variables	DFS		OS	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Tumor size >5 cm	1.054 (0.778-1.282)	0.832	1.113 (0.842-1.456)	0.548
Tumor distant Metastasis	1.764 (1.452-2.847)	0.001	1.483 (1.251-1.998)	0.011
High expression of Yap1 levels	1.692 (1.322-1.882)	0.012	1.758 (1.335-2.702)	0.001

cent nontumorous tissues (P<0.01, **Figure 2A-C**). Positive Yap1 IHC staining was observed in 18 patients (63.33%, **Figure 2D**).

In addition, the expression of LATS1, p-LATS1, Yap1 and p-Yap1 were also examined in four human osteosarcoma cells lines (HOS, Saos-2, U2OS, and MG-63) using Western blot. Yap1 and p-Yap1 expression increased in all OS cell lines. According to the comparison of Yap1 and p-Yap1 expression in OS cells, MG63 cells exhibited the highest expression of both Yap1 and p-Yap1 among four OS cell lines (**Figure 3**).

Upregulated expression of Yap1 is associated with aggressive clinicopathological features of patients with osteosarcoma

The clinicopathological parameters of patients with pediatric osteosarcoma were shown in **Table 1**. After further analysis, we found that Yap1 expression was associated with various clinicopathological parameters of osteosarcoma. In patients with large tumor size (>5 cm), Yap1 upregulation occurred significantly more often than those with small tumor size ≤5 cm, P<0.05). The elevated expression of Yap1 was also associated with the presence of metastasis (P<0.05). Taking into consideration the relationship between Yap1 expression and the response to pre-operative chemotherapy, we found that patients in high Yap1 expression group had poorer response to preoperative chemotherapy than those in low Yap1 expression group (P<0.05). However, no significant difference was observed between the expression of Yap1 and the gender and age of patients.

Upregulated expression of Yap1 conferred poor prognosis in patients with osteosarcoma

Using Kaplan-Meier method and log-rank test, we showed that the OS (**Figure 4A**, P=0.031) and PFS (**Figure 2B**, P=0.014) of patients with high Yap1 expression were both significantly shorter than those with low Yap1 expression. The favarable survival differences were also found in those with smaller tumor size (P<0.05), without metastasis (P<0.05).

After the the analysis by the Cox proportional hazard regressional model, we found that the Yap1 expression (for OS: RR 1.758, 95% CI, 1.335-2.702, P=0.001; for PFS: RR 1.692, 95%

CI, 1.322-1.882, P=0.012) and distant metastasis (for OS: RR 1.764, 95% CI, 1.452-2.847, P=0.001; for PFS: RR 1.483, 95% CI, 1.251-1.998, P=0.011) were independent prognostic factors of unfavorable survival in patients with osteosarcoma (**Table 2**). While tumor size (for OS: RR 1.054, 95% CI, 0.778-1.282, P=0.832; for PFS: RR 1.113, 95% CI, 0.842-1.456, P=0.548) was not an independent factor after multivariable adjustment.

Discussion

OS is the most common bone malignancy in children and adolescents, and it comprises about 3% of all pediatric tumors [27]. Despite aggressive multi-modality therapy applied, patients with advanced disease still have a poor prognosis with a 5-year survival rate at only 10 to 20% [28]. The poor prognosis of OS is associated with tumor invasion and metastasis, which often lead to therapeutic failure. Phosphorylation of YAP leads to its cytoplasmic retention and/or degradation, depending on the sites of phosphorylation [10, 29]. Conversely, loss of the Hippo signaling can lead to organ overgrowth and induce tumors in model organisms [30]. Dysregulation of the Hippo pathway occurs in a broad range of human carcinomas, including lung, colorectal, breast, ovarian, pancreatic, gastric, and liver cancer [31-34]. Although many reports have shown that the Hippo/YAP signaling pathway is involved in tumorigenesis, whether the Hippo pathway plays a role in the progression of osteosarcoma development is currently unknown. Previously many reports showed that high expression of YAP1 in osteosarcoma specimens with subsequently a higher expression of target genes related to the Hippo pathway [35, 36]. When YAP1 was knocked down by shRNA in MG-63 osteosarcoma cell line, proliferation and invasion were inhibited through inactivation of RUNX2 signaling. Additionally, tumor growth was decreased following YAP suppression in murine xenografts and in transgenic models. High expression of YAP1 by immunohistochemistry in a series of biopsies of osteosarcomas compared to normal bone was reported [37]. YAP1 expression was correlated with Enneking staging, with higher expression linked to stages II and III. Enneking staging is based on the tumor grade (low versus high), local extension and presence or absence of metastases.

In present study, we found that the expression of LATS1, p-LATS1, Yap1 and p-Yap1 were expressed highly in four human osteosarcoma cells lines (HOS, Saos-2, U2OS, and MG-63). Further analysis showed that Yap1 expression was associated tumor size (P<0.05), the presence of metastasis (P<0.05) and the response to pre-operative chemotherapy (P<0.05). patients with osteosarcoma with high Yap1 expression were both significantly shorter than those with low Yap1 expression. Overexpression of Yap1 was independent prognostic factors of unfavorable survival in pediatric osteosarcoma. after multivariable adjustment.

However, there are limitations of this study: (1) the sample size is too small in this study, and further larger sample study is needed to confirm the present experimental results; (2) whether overexpression of Yap1 have the optimal specificity and sensitivity for osteosarcoma diagnosis and prognosis also needs future confirmation.

In conclusion, we found Yap1 and other factors expressed significantly higher in osteosarcoma tissues and cell lines compared with non-tumorous tissues. Overexpression of Yap1 was an independently risk factor associated with the prognosis of patients osteosarcoma.

Disclosure of conflict of interest

None.

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