

ORIGINAL ARTICLE

Open Access



Hippo/YAP signaling pathway is involved in osteosarcoma chemoresistance

Dong-Yu Wang^{1†}, Ya-Nan Wu^{1†}, Jun-Qi Huang¹, Wei Wang¹, Meng Xu¹, Jin-Peng Jia¹, Gang Han¹, Bei-Bei Mao² and Wen-Zhi Bi^{1*}

Abstract

Background: Osteosarcoma is the most common bone malignancy in children and adolescents, and 20%–30% of the patients suffer from poor prognosis because of individual chemoresistance. The Hippo/yes-associated protein (YAP) signaling pathway has been shown to play a role in tumor chemoresistance, but no previous report has focused on its involvement in osteosarcoma chemoresistance. This study aimed to investigate the role of the Hippo/YAP signaling pathway in osteosarcoma chemoresistance and to determine potential treatment targets.

Methods: Using the Cell Titer-Glo Luminescent cell viability assay and flow cytometry analysis, we determined the proliferation and chemosensitivity of YAP-overexpressing and YAP-knockdown osteosarcoma cells. In addition, using western blotting and the real-time polymerase chain reaction technique, we investigated the alteration of the Hippo/YAP signaling pathway in osteosarcoma cells treated with chemotherapeutic agents.

Results: Mammalian sterile 20-like kinase 1 (MST1) degradation was increased, and large tumor suppressor kinase 1/2 (LATS1/2) total protein levels were decreased by methotrexate and doxorubicin, which increased activation and nuclear translocation of YAP. Moreover, YAP increased the proliferation and chemoresistance of MG63 cells.

Conclusions: The Hippo/YAP signaling pathway plays a role in osteosarcoma chemoresistance, and YAP is a potential target for reducing chemoresistance.

Keywords: Hippo, YAP, Methotrexate, Doxorubicin, Osteosarcoma, Chemoresistance

Background

Osteosarcoma is the most common bone malignancy in children and adolescents. Since the introduction of neoadjuvant chemotherapy (chemotherapy before treatment) in the 1980s, the prognosis of osteosarcoma patients has improved markedly [1]. However, in the past 10 years, the survival rate has risen only slightly. Currently, the consensus is that poor chemotherapeutic effect on some patients is the primary obstacle to a higher survival rate of osteosarcoma patients [2]. Methotrexate and doxorubicin are the most commonly used drugs for the treatment of osteosarcoma, and resistance to them substantially decreases patients' survival rates. Thus, many

studies have investigated the mechanism of chemoresistance to methotrexate and doxorubicin, including impaired intracellular transportation components [3], inactivation of chemotherapeutic drugs [4], DNA self-repair enhancements [5], cell signaling transduction turbulence [6], microRNA dysregulation [7], and autophagy overreaction [8]. Nevertheless, for patients with osteosarcoma, the key mechanism of chemoresistance is still inconclusive. This motivated us to investigate alternative mechanisms for osteosarcoma chemoresistance.

The Hippo/yes-associated protein (YAP) signaling pathway was originally found in the *Drosophila* and has been proven to modulate organ size [9]. Its key components include mammalian sterile 20-like kinases 1/2 (MST1/2), salvador family WW domain-containing protein 1 (SAV1), large tumor suppressor kinases 1/2 (LATS1/2), YAP, transcriptional co-activator with PDZ-binding motif (TAZ), and transcriptional enhancer

*Correspondence: biwenzhi@sina.com

[†]Dong-Yu Wang and Ya-Nan Wu contributed equally to this work

¹ Department of Orthopaedics and Rehabilitation, PLA General Hospital, Fuxing Rd 28, Beijing 100853, P. R. China

Full list of author information is available at the end of the article

factor domain family members 1–4 (TEAD1–4) [10]. In humans, MST1/2 combines with SAV1 to form an activated complex that initiates LATS1/2 phosphorylation [11–13]. Once activated, LATS1/2 further promotes the signaling cascade by phosphorylating YAP at Ser127 or TAZ at Ser89. Phosphorylated YAP then binds to 14-3-3 protein and remains in the cytoplasm for degradation [14–16]. Dephosphorylated YAP translocates into the nucleus and binds to TEAD1–4, which activates downstream genes to support proliferation and inhibit apoptosis [17, 18]. The Hippo/YAP signaling pathway is involved in tumor chemoresistance. Mao et al. [19] reported that resistance to cisplatin is increased by YAP2 and silent mating type information regulation 2 homolog 1 (SIRT1) in hepatocellular carcinoma (HCC) cells, indicating that both YAP2 and SIRT1 protect HCC cells from the chemotherapeutic drug cisplatin. Similarly, ovarian cancer cells with knockdown of YAP/TEAD showed increased sensitivity to cisplatin, paclitaxel, and bleomycin [20]. Moreover, verteporfin, a YAP1 inhibitor, promotes sensitivity to 5-fluorouracil and docetaxel by directly inhibiting YAP1 and endothelial growth factor receptor in esophageal cancer cells [21]. Although many studies have investigated the role of the Hippo/YAP signaling pathway in chemoresistance, little is known about its function in osteosarcoma chemoresistance.

In this study, we try to find the role of Hippo/YAP signaling pathway in methotrexate- or doxorubicin-treated MG63 and U2OS osteosarcoma cells. We hope our experiments illustrate the function of YAP in osteosarcoma chemoresistance.

Methods

Cell cultures and reagents

Human osteosarcoma cell lines MG63 and U2OS were purchased from Cell Resource Center of Shanghai Institutes for Biological Sciences (Shanghai, China) and cultured in Minimal Essential Medium (Gibco, Waltham, Massachusetts, USA) with 10% fetal bovine serum (Biological Industries, Kibbutz Beit Haemek, Israel), 1% non-essential amino acid (Gibco), and penicillin/streptomycin (Gibco) in a humidified incubator under 95% air and 5% CO₂ at 37 °C. All other cell culture materials were obtained from Gibco; all chemicals were obtained from Sigma-Aldrich (St. Louis, Missouri, USA).

Virus packaging and infection

pQCXIH empty vector and pQCXIH-YAP constructs were gifts from Bin Zhao (Zhejiang University, China) [18]. pLKO empty vector and pLKO-YAP-knockdown expressing lentivirus were also constructed to obtain YAP knockdown cell lines. MG63 cells were infected with retrovirus that expresses empty vector and wild-type (WT)

YAP separately to generate control and YAP-overexpressing stable cell lines. pLKO empty vector and pLKO-YAP-knockdown expressing lentivirus were used to treat MG63 cells to generate control and YAP-knockdown stable cell lines. Hygromycin and blasticidin screening was performed 48 h after infection.

RNA extraction and quantitative real-time polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated from cells using TRIzol reagent (Invitrogen-Life Technologies, Waltham, Massachusetts, USA). The reverse transcription products were used for RT-PCR with specific primers: MST1 (forward: 5'-AGACCTCCAGGAGATAATCAAAGA-3'; reverse: 5'-AGATACAGAACCAGCCCCACA-3'), Beta-Actin (forward: 5'-GTCTGCCTTGGTAGTGGATAATG-3'; reverse: 5'-TCGAGGACGCCCTATCATGG-3').

Immunofluorescence staining

MG63 and U2OS cells were fixed using 4% paraformaldehyde in phosphate buffered saline (PBS) for 15 min. After permeabilization, using 0.1% Triton X-100 in PBS and blocking in 3% bovine serum albumin in PBS, the cells were incubated in primary antibodies overnight at 4 °C. Alexa Fluor 546-conjugated secondary antibodies (Invitrogen-Life Technologies; 1:1000 dilution) were used. The samples were mounted using ProLong Gold Antifade Reagent with DAPI (Invitrogen-Life Technologies), and immunofluorescence was detected using an Olympus confocal microscope.

Co-immunoprecipitation

Cells were collected, and proteins were solubilized in immunoprecipitation buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% NP40, 1% protease inhibitor cocktail) at 4 °C. Then, 1 mg of lysed protein was incubated with YAP antibody (ABclonal Biotech, A1002, College Park, Maryland, USA) and precipitated with protein A or G agarose (Upstate Biotechnology, Lake Placid, New York, USA) at 4 °C overnight. The immune complex was collected, washed three to five times, and probed with 14-3-3 β antibody (Cell Signaling Technology, #9636, Danvers, Massachusetts, USA) and YAP antibody (ABclonal Biotech).

Cell counting

MG63 cells were cultured in 96-well flat plates for 6 days. Before seeding, cell numbers were calculated using a countess automated cell counter (Invitrogen-Life Technologies) to keep the initial cell numbers equal. Culture media were rejuvenated every 48 h, and total cell numbers of cells were counted every 24 h. In this study, three independent experiments were performed.

Cell viability assay

Cell Titer-Glo Luminescent Cell Viability Assay (Promega, Madison, Wisconsin, USA) was used to monitor cell total adenosine triphosphate (ATP). MG63 cells were seeded in a 96-well flat plate for 24 h and exposed to methotrexate (20 mM) or doxorubicin (10 μ M) for another 24 h. Then, the Cell Titer-Glo reagent was added to the cells for 10 min. ATP was measured using a reporter luminometer. Relative cell viability was calculated according to the manufacturer's instructions. This experiment was repeated three times.

Cell apoptosis assay

Cell apoptosis was examined by flow cytometry analysis using the Annexin V-FITC and propidium iodide (PI) double-staining technique. MG63 cells were seeded in a 24-well culture plate at greater than 80% confluence and subjected to methotrexate (20 mM) or doxorubicin (10 μ M) treatments for 24 h. Cells were stained following the Annexin V-fluorescein isothiocyanate (FITC) cell apoptosis detection kit's instructions (Beyotime Biotechnology, C1062, Shanghai, China). To confirm our results, three independent experiments were conducted.

Western blotting

Cells were lysed in a RIPA buffer (Beyotime Biotechnology), and total protein concentration was measured using a BIO-RAD Quick Start Bradford Dye Reagent (#500-0205; Bio-Rad Laboratories, Hercules, California, USA) according to the manufacturer's instructions. Western blotting procedures were performed as reported previously [22]. Grayscale analysis was conducted using Image J software (National Institute of Health, Bethesda, Maryland, USA), and results were calculated from three independent experiments. The primary antibodies used in our experiments were as follows: YAP (ABclonal Biotech, A1002), Phospho-YAP (Cell Signaling Technology, #13008), LATS2 (Sigma-Aldrich, WH0007004M1), LATS1 (Bethyl laboratory, A300-477A; Montgomery, Texas, USA), MST1 (Cell Signaling Technology, #3682), 14-3-3 β (Cell Signaling Technology, #9636), and GAPDH (Cell Signaling Technology, #5174).

Statistical analysis

Results are presented as mean \pm standard deviation. Comparisons between two groups were assessed using the unpaired Student's *t* test. Cyclohexamide grayscale comparison was made using the paired *t* test. *P* values less than 0.05 were considered statistically significant. All statistical analyses were conducted using GraphPad Prism software (GraphPad Software, San Diego, California, USA).

Results

YAP regulated the proliferation and chemoresistance of osteosarcoma cells

To investigate the function of the Hippo/YAP pathway in osteosarcoma chemoresistance, we successfully established stable YAP-overexpressing and YAP-knockdown MG63 cell lines by retrovirus and lentiviral infection. As shown in Fig. 1a, overexpressing and knockdown of YAP resulted in accelerated and slowed cell proliferation, as detected by cell number counting. Moreover, cell viability assay showed that overexpression of YAP increased the viability of MG63 cells treated with high-concentration methotrexate (20 mM) or doxorubicin (10 μ M) (Fig. 1b). Annexin V-FITC/PI staining and flow cytometry analysis confirmed the protective function of YAP in response to methotrexate (20 mM) or doxorubicin (10 μ M), as the apoptosis of YAP-overexpressing cells was significantly lower than that of the control (*P* = 0.001 and *P* = 0.043, respectively). Additionally, YAP-knockdown cells demonstrated increased sensitivity to methotrexate and doxorubicin (Fig. 1c). Together, these data showed that YAP increased cell growth and the chemoresistance of osteosarcoma cells.

Methotrexate and doxorubicin induced YAP activation in MG63 and U2OS osteosarcoma cells

To further investigate the role of the Hippo/YAP pathway in osteosarcoma chemoresistance, we evaluated LATS1/2 total protein level and Ser127 phosphorylation of YAP in osteosarcoma cells. Before Western blotting analysis, MG63 and U2OS were treated with methotrexate or doxorubicin at different concentrations for 24 h. We observed that LATS1/2 total protein decreased in osteosarcoma cells treated with methotrexate or doxorubicin (Figs. 2, 3). As shown in Fig. 2a and c, phosphorylation of YAP decreased significantly in a concentration-dependent manner after doxorubicin treatment. Similar results were found after treatment of methotrexate (Fig. 2b, d). Grayscale comparison of western blotting results showed that both methotrexate and doxorubicin could induce YAP activation, suggesting that YAP plays a role in osteosarcoma chemoresistance.

Methotrexate and doxorubicin induced YAP nuclear translocation

Since YAP phosphorylation at Ser127 determines its location in either the cytoplasm or the nucleus [23], using immunofluorescence staining we examined the intracellular location of YAP in methotrexate- or doxorubicin-treated MG63 and U2OS cells. We found that YAP translocated to the nucleus in both MG63 and U2OS cells after methotrexate or doxorubicin treatment (Fig. 4a, b). 14-3-3 protein is well known for its critical

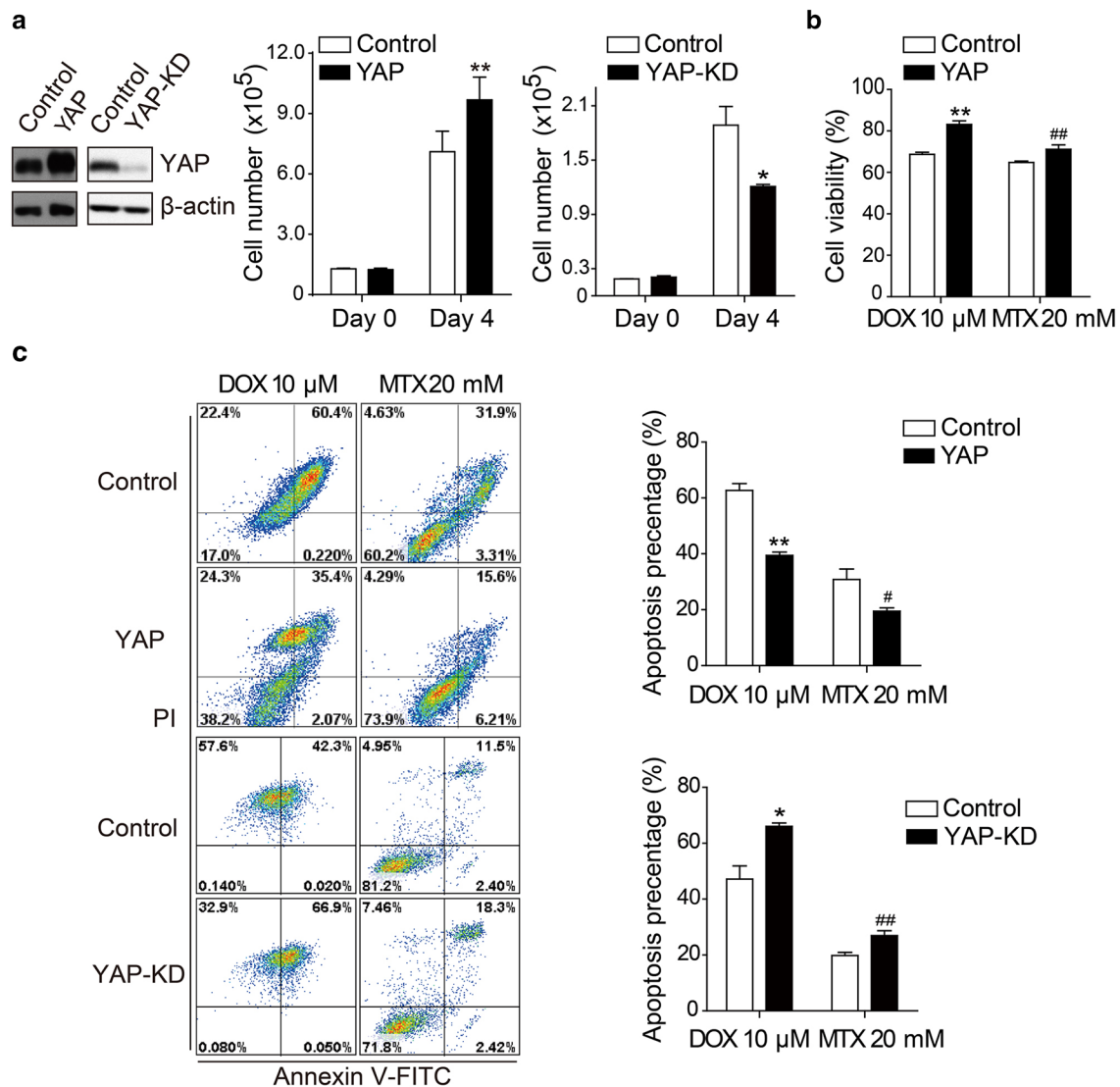
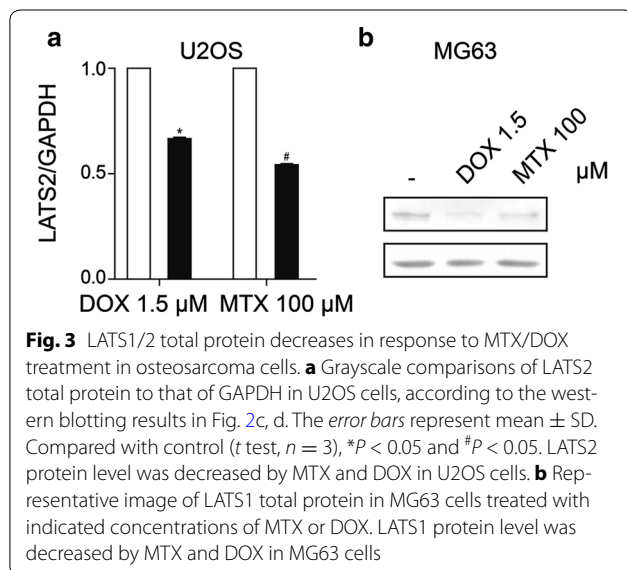
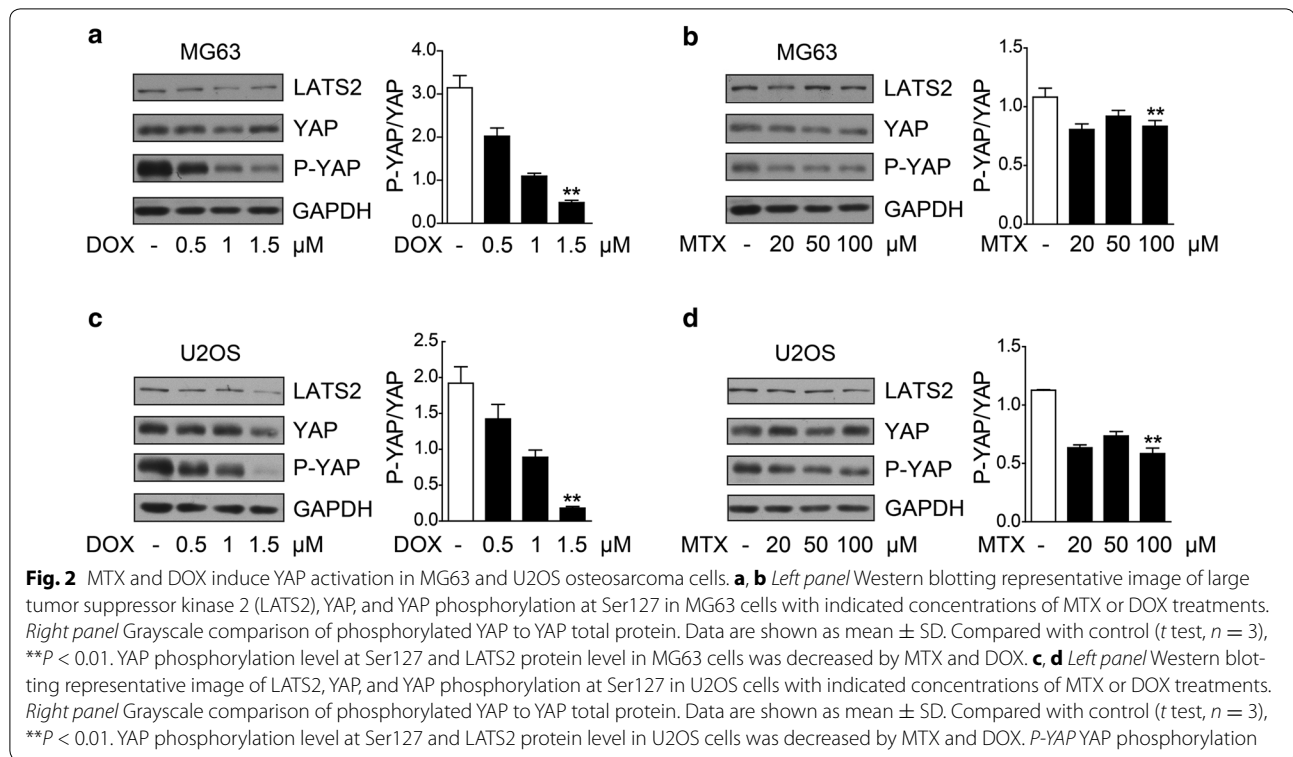


Fig. 1 Yes-associated protein (YAP) increases the proliferation and chemoresistance of MG63 osteosarcoma cells. **a** Control and YAP-overexpressing/knockdown MG63 cells were seeded at the same concentration, and cell numbers were counted every 24 h. Data are shown as mean \pm standard deviation (SD). Compared with control (t test, $n = 3$), $**P < 0.01$, and $*P < 0.05$. Results show that overexpression of YAP accelerated MG63 cell proliferation and knockdown of YAP decreased cell proliferation. **b** Cell viability was analyzed by detecting total cellular adenosine triphosphate in methotrexate (MTX) (20 mM)- or doxorubicin (DOX) (10 μ M)-treated control and YAP-overexpressing MG63 cells. Data are shown as mean \pm SD. Compared with control (t test, $n = 3$), $**P < 0.01$, and $##P < 0.01$. Overexpression of YAP increased the viability of MG63 cells treated with MTX (20 mM) or DOX (10 μ M). **c** *Left panel* representative images of flow cytometry analysis of YAP-overexpressing and YAP-knockdown MG63 cells treated with MTX (20 mM) or DOX (10 μ M) and stained by Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide. Due to the natural fluorescence of DOX, we calculated only Annexin V-FITC-positive cells in DOX-treated cells. *Right panel* Quantitative analysis of apoptosis percentages according to the results of *left panel*. Data are shown as mean \pm SD. Compared with control (t test, $n = 3$), $*P < 0.05$, $**P < 0.01$, $##P < 0.05$, and $###P < 0.01$. The flow cytometry results showed that YAP increased the chemoresistance of osteosarcoma cells and knockdown of YAP increased the chemosensitivity of osteosarcoma cells. YAP-KD, knockdown of YAP

role in inhibiting the nuclear translocation of YAP. To further validate the effect of methotrexate and doxorubicin on YAP activity, we determined the interaction between YAP and 14-3-3. Cells exposed to methotrexate or doxorubicin were harvested for co-immunoprecipitation studies. Consistent with the results from the

immunofluorescence staining assays, methotrexate and doxorubicin dramatically reduced the interaction between YAP and 14-3-3 (Fig. 4c, d). Methotrexate and doxorubicin decreased the interaction between YAP and 14-3-3 β and induced YAP nuclear translocation, indicating that both are capable of activating YAP.



Methotrexate and doxorubicin decreased MST1 expression by altering its protein stability

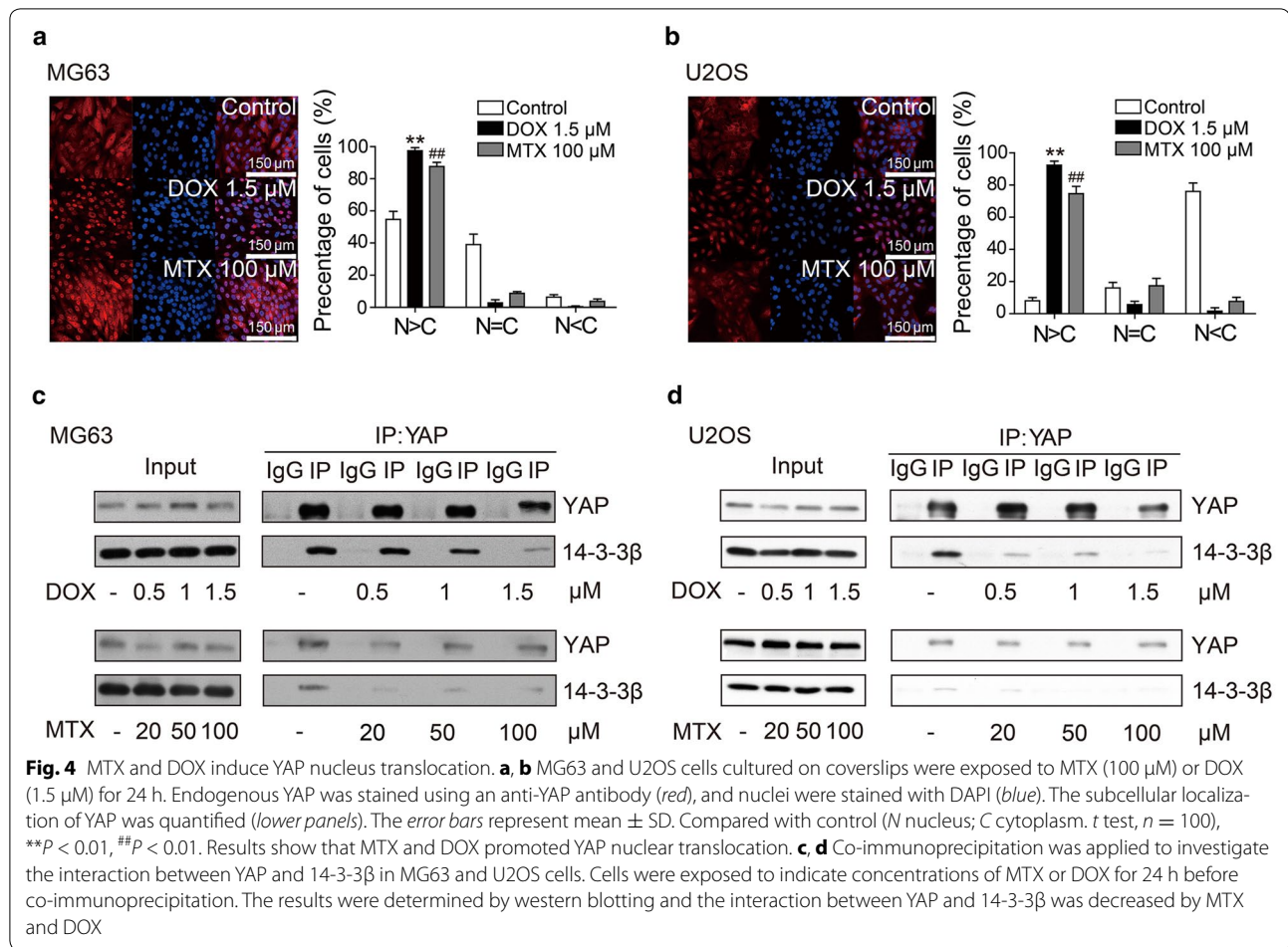
MST1 is a key component of the Hippo signaling pathway. To better understand MST1's role in osteosarcoma chemoresistance, we examined its protein level. As shown in Fig. 5a, total protein of MST1 remarkably declined in methotrexate- or doxorubicin-treated osteosarcoma cells.

However, after methotrexate and doxorubicin treatment, we did not observe the down-regulation of MST1 mRNA level (Fig. 5b), suggesting that doxorubicin and methotrexate reduce MST1 expression at the protein level. Then, U2OS cells were treated with cycloheximide (a common inhibitor of protein biosynthesis in eukaryotic organisms) for the times indicated and harvested for analysis of the MST1 protein level. As shown in Fig. 5c, methotrexate and doxorubicin accelerated degradation of MST1, suggesting that MST1 is destabilized by methotrexate and doxorubicin, which is responsible for the activation of YAP induced by methotrexate and doxorubicin.

Discussion

Although many reports have shown that the Hippo/YAP signaling pathway is involved in tumorigenesis, little is known about its role in osteosarcoma chemoresistance. In the present study, we showed that, in osteosarcoma cells, methotrexate and doxorubicin activated YAP, promoting its nuclear translocation by accelerating MST1 protein degradation and decreasing LATS1/2 protein level. Furthermore, YAP regulated the proliferation and chemoresistance in MG63 osteosarcoma cells, indicating that the Hippo/YAP pathway plays a role in osteosarcoma chemoresistance (Fig. 6).

Current management of osteosarcoma patients focuses on neoadjuvant chemotherapy plus surgery. However,



many patients die from tumor metastases because of poor response to chemotherapy. In the past 10 years, several cell signaling pathways, including phosphoinositide 3 kinase (PI3 K)/Akt, extracellular signal-regulated kinase (ERK)1/2, Notch, and Wnt- β -catenin, have been identified to be involved in osteosarcoma chemoresistance [24–26]. Recently, the Hippo/YAP signaling pathway has been shown to modulate organ size [9, 27]. Moreover, other studies have shown that YAP promotes neoplastic cell proliferation and accelerates oncogenic senescence [28, 29]. Mao et al. [19] showed that SIRT1 increases the interaction between YAP2 and TEAD4 and enhances resistance to the anti-cancer drug cisplatin by deacetylating YAP2 in HCC cells. Phosphorylation-defective YAP overexpression makes ovarian cancer cells much more resistant to cisplatin [30]. Nevertheless, the relationship between osteosarcoma chemoresistance and the Hippo/YAP signaling pathway is still unclear. To our knowledge, our study is the first to focus on the function of the Hippo/YAP signaling pathway in osteosarcoma chemoresistance. We found that, with methotrexate and doxorubicin treatment, YAP increases MG63 cell proliferation and cytotoxic survivability.

Accordingly, methotrexate and doxorubicin inhibit the phosphorylation of YAP. Similar to doxorubicin, cisplatin also inhibits YAP phosphorylation at Ser 127 in HCC cells [19]. Activated YAP then translocates to the nucleus and enhances the chemoresistance of osteosarcoma cells.

We also determined that reduced MST1 and LATS1/2 protein level in response to methotrexate and doxorubicin may cause up-regulation of YAP activity. Previously, our colleagues [31] reported that c-Abl stabilizes MST1 protein level and protects it from ubiquitination by phosphorylating MST1 at Y433 in HEK 293T and Neuro2A cells. In addition, Ren et al. [32] found that proteasome-mediated down-regulation of MST1 by heat shock protein 70 enhances resistance to cisplatin in prostate cancer cells. Autophagy is another regulated pathway of cellular degradation. In various tumor cells, increased autophagy has shown protective effects against cytotoxic agents [33, 34]. MST1/2 directly phosphorylates LC3 and enhances the cell autophagy process [35]. Therefore, decrease of MST1 could decrease cell autophagy and then improve osteosarcoma chemosensitivity. As there is no evidence for lysosome-mediated degradation of MST1, we could

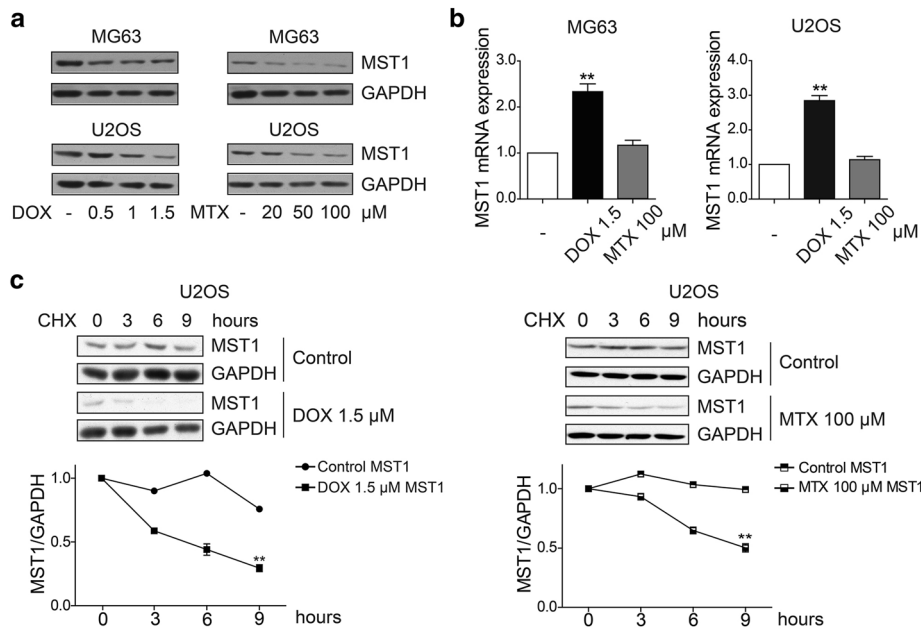


Fig. 5 MTX and DOX decrease mammalian sterile 20-like kinase 1 (MST1) expression by altering its protein stability. **a** Western blotting representative image of mammalian sterile 20-like kinase 1 (MST1) total protein level in MTX- or DOX-treated osteosarcoma cells. MST1 protein level was decreased by MTX and DOX. **b** The MST1 mRNA levels in the control and MTX- or DOX-treated osteosarcoma cells were monitored via real-time polymerase chain reaction using specific primers. The error bars represent mean \pm SD. Compared with control (*t* test, *n* = 3), $^{***}P < 0.01$. MST1 mRNA did not decrease after MTX or DOX treatments. **c** U2OS cells were treated with 100 μ g/mL of cycloheximide for the indicated periods. The endogenous MST1 protein levels were determined by western blotting (upper panels), and relative MST1 protein levels were normalized to those of GAPDH (lower panels). The error bars represent mean \pm SD. Compared with control (paired *t* test, *n* = 3), $^{***}P < 0.01$. MST1 degradation was increased by MTX and DOX in U2OS cells. CHX cycloheximide

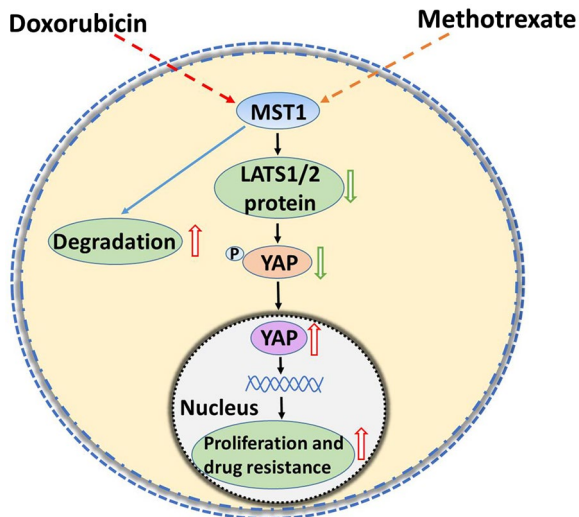


Fig. 6 Systematic model of the Hippo/YAP signaling pathway affecting cytotoxic drug resistance in osteosarcoma cells. Methotrexate and doxorubicin increase MST1 degradation in osteosarcoma cells, decreasing LATS1/2 total protein level and YAP phosphorylation, resulting in enhanced nuclear translocation of YAP. Endonuclear YAP then promotes the transcription of its downstream targets involved in anti-apoptosis and proliferation, leading to elevated proliferation and resistance to methotrexate and doxorubicin

not confirm whether proteasomal or lysosomal degradation is responsible for the decrease of MST1 protein level in osteosarcoma cells treated with chemotherapeutic drugs. Further experiments are needed to address this.

Conclusions

In conclusion, our results suggest that the Hippo/YAP signaling pathway induces osteosarcoma chemoresistance. The reduction in the concentration of MST1 and LATS1/2 proteins by methotrexate and doxorubicin leads to YAP activation and nuclear translocation. Moreover, YAP increases the proliferation and methotrexate/doxorubicin resistance in MG63 cells. Taken together, our findings suggest that the decrease of YAP may improve osteosarcoma chemosensitivity.

Authors' contributions

BBM and WZB conceived of this project. DYW and YNW conducted experiments, and DYW drafted the manuscript. DYW, JQH, and XM performed statistical analyses. WW, JPJ, and HG provided material and discussed the results. All authors read and approved the final manuscript.

Author details

¹ Department of Orthopaedics and Rehabilitation, PLA General Hospital, Fuxing Rd 28, Beijing 100853, P. R. China. ² State Key Laboratory of Brain and Cognitive Sciences, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, P. R. China.

Acknowledgements

We sincerely thank Bin Zhao of Zhejiang University for the pQCXIH empty vector and pQCXIH-YAP constructs. This work was supported by the National Natural Science Foundation of China (No. 81172553 and 81472513 to WB).

Competing interests

The authors declare that they have no competing interests.

Received: 29 July 2015 Accepted: 3 May 2016

Published online: 20 May 2016

References

- Rosen G, Caparros B, Huvos AG, Kosloff C, Nirenberg A, Cacavio A, et al. Preoperative chemotherapy for osteogenic sarcoma: selection of post-operative adjuvant chemotherapy based on the response of the primary tumor to preoperative chemotherapy. *Cancer*. 1982;49(6):1221–30.
- Bielack SS, Kempf-Bielack B, Delling G, Exner GU, Flege S, Helmke K, et al. Prognostic factors in high-grade osteosarcoma of the extremities or trunk: an analysis of 1702 patients treated on neoadjuvant cooperative osteosarcoma study group protocols. *J Clin Oncol*. 2002;20(3):776–90.
- Hattinger CM, Reverter-Branchat G, Remondini D, Castellani GC, Benini S, Pasello M, et al. Genomic imbalances associated with methotrexate resistance in human osteosarcoma cell lines detected by comparative genomic hybridization-based techniques. *Eur J Cell Biol*. 2003;82(9):483–93. doi:10.1078/0171-9335-00336.
- Townsend DM, Tew KD. The role of glutathione-S-transferase in anti-cancer drug resistance. *Oncogene*. 2003;22(47):7369–75. doi:10.1038/sj.onc.1206940.
- Yang JL. Investigation of osteosarcoma genomics and its impact on targeted therapy: an international collaboration to conquer human osteosarcoma. *Chin J Cancer*. 2014;33(12):575–80. doi:10.5732/cjc.014.10209.
- Gazitt Y, Kolaparthy V, Moncada K, Thomas C, Freeman J. Targeted therapy of human osteosarcoma with 17AAG or rapamycin: characterization of induced apoptosis and inhibition of mTOR and Akt/MAPK/Wnt pathways. *Int J Oncol*. 2009;34(2):551–61.
- Cai CK, Zhao GY, Tian LY, Liu L, Yan K, Ma YL, et al. miR-15a and miR-16-1 downregulate CCND1 and induce apoptosis and cell cycle arrest in osteosarcoma. *Oncol Rep*. 2012;28(5):1764–70. doi:10.3892/or.2012.1995.
- Kim HJ, Lee SG, Kim YJ, Park JE, Lee KY, Yoo YH, et al. Cytoprotective role of autophagy during paclitaxel-induced apoptosis in Saos-2 osteosarcoma cells. *Int J Oncol*. 2013;42(6):1985–92. doi:10.3892/ijo.2013.1884.
- Udan RS, Kango-Singh M, Nolo R, Tao C, Halder G. Hippo promotes proliferation arrest and apoptosis in the Salvador/Warts pathway. *Nat Cell Biol*. 2003;5(10):914–20. doi:10.1038/ncb1050.
- Kodaka M, Hata Y. The mammalian Hippo pathway: regulation and function of YAP1 and TAZ. *Cell Mol Life Sci*. 2015;72(2):285–306. doi:10.1007/s00018-014-1742-9.
- Basu S, Totty NF, Irwin MS, Sudol M, Downward J. Akt phosphorylates the Yes-associated protein, YAP, to induce interaction with 14-3-3 and attenuation of p73-mediated apoptosis. *Mol Cell*. 2003;11(1):11–23.
- Dong J, Feldmann G, Huang J, Wu S, Zhang N, Comerford SA, et al. Elucidation of a universal size-control mechanism in *Drosophila* and mammals. *Cell*. 2007;130(6):1120–33. doi:10.1016/j.cell.2007.07.019.
- Qin F, Tian J, Zhou D, Chen L. Mst1 and Mst2 kinases: regulations and diseases. *Cell Biosci*. 2013;3(1):31. doi:10.1186/2045-3701-3-31.
- Hao Y, Chun A, Cheung K, Rashidi B, Yang X. Tumor suppressor LATS1 is a negative regulator of oncogene YAP. *J Biol Chem*. 2008;283(9):5496–509. doi:10.1074/jbc.M709037200.
- Oh H, Irvine KD. In vivo regulation of Yorkie phosphorylation and localization. *Development*. 2008;135(6):1081–8. doi:10.1242/dev.015255.
- Oka T, Mazack V, Sudol M. Mst2 and Lats kinases regulate apoptotic function of Yes kinase-associated protein (YAP). *J Biol Chem*. 2008;283(41):27534–46. doi:10.1074/jbc.M804380200.
- Vassilev A, Kaneko KJ, Shu H, Zhao Y, DePamphilis ML. TEAD/TEF transcription factors utilize the activation domain of YAP65, a Src/Yes-associated protein localized in the cytoplasm. *Genes Dev*. 2001;15(10):1229–41. doi:10.1101/gad.888601.
- Zhao B, Ye X, Yu J, Li L, Li W, Li S, et al. TEAD mediates YAP-dependent gene induction and growth control. *Genes Dev*. 2008;22(14):1962–71. doi:10.1101/gad.1664408.
- Mao B, Hu F, Cheng J, Wang P, Xu M, Yuan F, et al. SIRT1 regulates YAP2-mediated cell proliferation and chemoresistance in hepatocellular carcinoma. *Oncogene*. 2014;33(11):1468–74. doi:10.1038/onc.2013.88.
- Xia Y, Zhang YL, Yu C, Chang T, Fan HY. YAP/TEAD co-activator regulated pluripotency and chemoresistance in ovarian cancer initiated cells. *PLoS ONE*. 2014;9(11):e109575. doi:10.1371/journal.pone.0109575.
- Song S, Honjo S, Jin J, Chang SS, Scott AW, Chen Q, et al. The Hippo coactivator YAP1 mediates EGFR overexpression and confers chemoresistance in esophageal cancer. *Clin Cancer Res*. 2015;21(11):2580–90. doi:10.1158/1078-0432.CCR-14-2191.
- Liao Y, Hao Y, Chen H, He Q, Yuan Z, Cheng J. Mitochondrial calcium uniporter protein MCU is involved in oxidative stress-induced cell death. *Protein Cell*. 2015;6(6):434–42. doi:10.1007/s13238-015-0144-6.
- Ramos A, Camargo FD. The Hippo signaling pathway and stem cell biology. *Trends Cell Biol*. 2012;22(7):339–46. doi:10.1016/j.tcb.2012.04.006.
- Huang H, Wang L, Li M, Wang X, Zhang L. Secreted clusterin (sCLU) regulates cell proliferation and chemosensitivity to cisplatin by modulating ERK1/2 signals in human osteosarcoma cells. *World J Surg Oncol*. 2014;12:255. doi:10.1186/1477-7819-12-255.
- Ma Y, Ren Y, Han EQ, Li H, Chen D, Jacobs JJ, et al. Inhibition of the Wnt-beta-catenin and Notch signaling pathways sensitizes osteosarcoma cells to chemotherapy. *Biochem Biophys Res Commun*. 2013;431(2):274–9. doi:10.1016/j.bbrc.2012.12.118.
- Zhang J, Yu XH, Yan YG, Wang C, Wang WJ. PI3 K/Akt signaling in osteosarcoma. *Clin Chim Acta*. 2015;444:182–92. doi:10.1016/j.cca.2014.12.041.
- Zhao B, Li L, Lei Q, Guan KL. The Hippo-YAP pathway in organ size control and tumorigenesis: an updated version. *Genes Dev*. 2010;24(9):862–74. doi:10.1101/gad.1909210.
- Fausti F, Di Agostino S, Ciocce M, Bielli P, Sette C, Pandolfi PP, et al. ATM kinase enables the functional axis of YAP, PML and p53 to ameliorate loss of Werner protein-mediated oncogenic senescence. *Cell Death Differ*. 2013;20(11):1498–509. doi:10.1038/cdd.2013.101.
- Hiemer SE, Zhang L, Kartha VK, Packer TS, Almershed M, Noonan V, et al. A YAP/TAZ-regulated molecular signature is associated with oral squamous cell carcinoma. *Mol Cancer Res*. 2015;13(6):957–68. doi:10.1158/1541-7786.MCR-14-0580.
- Hall CA, Wang R, Miao J, Oliva E, Shen X, Wheeler T, et al. Hippo pathway effector Yap is an ovarian cancer oncogene. *Cancer Res*. 2010;70(21):8517–25. doi:10.1158/0008-5472.CAN-10-1242.
- Xiao L, Chen D, Hu P, Wu J, Liu W, Zhao Y, et al. The c-Abl-MST1 signaling pathway mediates oxidative stress-induced neuronal cell death. *J Neurosci*. 2011;31(26):9611–9. doi:10.1523/JNEUROSCI.0035-11.2011.
- Ren A, Yan G, You B, Sun J. Down-regulation of mammalian sterile 20-like kinase 1 by heat shock protein 70 mediates cisplatin resistance in prostate cancer cells. *Cancer Res*. 2008;68(7):2266–74. doi:10.1158/0008-5472.CAN-07-6248.
- Kong Q, Xu LH, Xu W, Fang JP, Xu HG. HMGB1 translocation is involved in the transformation of autophagy complexes and promotes chemoresistance in leukaemia. *Int J Oncol*. 2015;47(1):161–70. doi:10.3892/ijo.2015.2985.
- Tan Q, Wang H, Hu Y, Hu M, Li X, Aodeng Q, et al. Src/STAT3-dependent HO-1 induction mediates chemoresistance of breast cancer cells to doxorubicin by promoting autophagy. *Cancer Sci*. 2015;106(8):1023–32. doi:10.1111/cas.12712.
- Wilkinson DS, Hansen M. LC3 is a novel substrate for the mammalian Hippo kinases, STK3/STK4. *Autophagy*. 2015;11(5):856–7. doi:10.1080/1548627.2015.1017197.