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Original Article

LATS2-mediated YAP1 phosphorylation is involved in HCC tumorigenesis

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Abstract: YAP (yes-associated protein) is a transcriptional co-activator that acts downstream of the Hippo signaling pathway and regulates multiple cellular processes, including proliferation and apoptosis. Although YAP plays an important role in various tumors, the underlying mechanism in hepatocellular carcinoma (HCC) tumorigenesis remains unclear. In this study, we observed that the LATS2 was highly expressed in Bel-7402 and HepG2 cell lines, and LATS2 protein level was negatively correlated with YAP1 in HCC cells. And then, we inhibited LATS2 expression by transfecting with siRNA. Western blot and Immunofluorescent staining analysis demonstrated that LATS2 inhibition decreased the dephosphorylation of YAP1 protein and promoted YAP1 nuclear accumulation in HCC cells. Moreover, Immunoprecipitation assay results also indicated that Yap binds directly to TEAD2 and LATS2 inhibition-mediated dephosphorylation increased the YAP1/TEAD2 association, leading to YAP1/TEAD2 transcriptional activation, which in turn upregulated cell invasion in HCC cells. Taken together, our current data indicated a new regulatory mechanism of YAP1 by the LATS2-mediated phosphorylation that was involved in HCC tumorigenesis.

Keywords: Hepatocellular carcinoma, LATS2, YAP1, TEAD2, phosphorylation

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies and ranks third among the most lethal cancers worldwide [1]. HCC has a poor prognosis with about 5% of patients surviving more than 5 years [2]. So it is urgent and absolutely necessary *for* us to have a better understanding of the molecular pathogenesis of HCC.

The Hippo signaling pathway plays a critical role in oncogenesis by regulating cell proliferation, epithelial-mesenchymal transition, and apoptosis. LATS2, human large tumor suppressor 2 (also known as KPM), encodes a putative Ser/Thr protein kinase and plays a critical role in mediating Hippo (Hpo) growth inhibitory signaling [3]. Dysregulation of LATS2 expression has been shown to participate in both the onset and progression of various malignant tumors [4-6]. In breast cancer, LATS2 mRNA expression was down-regulated by promoter hypermethylation, which was associated with large tumor size, high rate of metastasis and estrogen receptor and progesterone receptor negativity [7]. Reduced LATS2 expression occurred in prostate tumors and LATS2 negatively modulated androgen receptor-regulated gene transcription, indicating that LATS2 may also play important role on prostate cancer progression [8]. Recent study showed that down-regulated LATS and increased expression of yes-associated protein (Yap) were observed in human hepatocellular carcinoma [9]. However, the molecular mechanism by which LATS2 on HCC metastasis is unclear at present.

The downstream effector of the pathway is a transcriptional coactivator namely yes-associated protein (Yap) [10]. Recent studies identified that YAP as an oncogene candidate or a tumor suppressor plays an important role in various tumors. For example, in clinical specimens, overexpression and nuclear accumulation of YAP have been reported in prostate, colon, breast, non-small-cell lung cancer, as well as in ovarian and liver cancers [11, 12]. YAP1 accelerated tumorigenesis and were required to sustain rapid growth of amplicon-containing tumors in mouse liver carcinoma [13]. Furthermore, YAP transgenic mice demon-

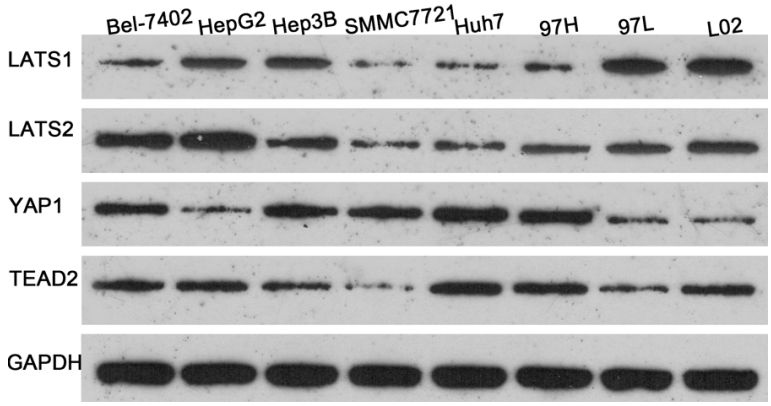

Figure 1. Western blot analysis revealed the expression of LATS1, LATS2, YAP1 and TEAD2 in human HCC and embryo liver cell lines.

strated a remarkable increase in liver tumors progression [10, 14]. Clinical study in a cohort of 177 HCC patients further demonstrated that YAP is an independent prognostic marker associated with poor disease free survival and overall survival in HCC [15]. Yap partners with transcription factor TEAD to initiate number of genes involved in cell proliferation and apoptosis including survivin (BIRC5), CTGF and cyclin D1 [16]. TEAD is critical for YAP's oncogenic activity and promotes YAP-mediated tumorigenesis in tumors [17, 18].

In this study, we demonstrated that LATS2 inhibition decreased YAP1 phosphorylation. The dephosphorylation of YAP1 promoted YAP1 nuclear accumulation and upregulated the YAP1/TEAD2 association, leading to YAP1/TEAD2 transcriptional activation and cell invasion in HCC cells. Our findings revealed a new regulatory mechanism of YAP1 by the LATS2-mediated phosphorylation that may be involved in liver tumorigenesis.

Materials and methods

Cell culture and reagents

Bel-7402, HepG2, Hep3B, SMMC7721, Huh7, 97H, 97L and L02 cells were maintained in Dulbecco's modified Eagle's medium supplemented with glutamine, penicillin/streptomycin, and 10% fetal bovine serum. 293T, Bel-7402 and HepG2 cells were transfected with lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Anti-LATS1, Anti-LATS2, Anti-YAP1 (ab81183), Anti-YAP1 (phospho Y357) and Anti-YAP1 (phospho S127) antibody were purchased from Sigma-Aldrich. Anti-TEAD2 antibody was purchased from SANTA.

Transfection of HepG2 cells with siRNAs

The siRNAs were designed and synthesized by GenePharma. Four siRNAs that were synthesized to target LATS2 expression were used in this experiment: siRNA-1 (LATS2-homo-403) sense 5'-UACCAUAAAUACAAUCUUCTT; siRNA-2 (LATS2-homo-643) sense 5'-UGAUAAGGUCCGAACUUUGTT; siRNA-3 (LATS2-homo-3821) sense 5'-AAUUUCAAGUGAAGUAAUCTT; Scramble control siRNAs sense 5'-UUCUCCGAACGUGUCACGUTT. siRNAs transfection was performed using Lipofectamine 2000 (Invitrogen). Total protein assay was prepared 72 h after transfection for protein gel blot analysis.

Immunoprecipitation and immunoblotting

Cell lysates were incubated with the indicated antibodies in the presence of 15 μl of protein G-agarose beads for 3 hours at 4°C. After washed five times, the immunoprecipitates were subjected to electrophoresis. Protein expression was examined by probing Western blots of total cell lysates or immunoprecipitates with the appropriate antibodies as noted in the figure legends.

Immunofluorenscence

Cultured cells were grown on glass coverslips, washed with PBS and fixed in 4% paraformaldehyde. After permeabilization with 0.1% Triton X-100 in PBS, cells were blocked in 3% BSA in PBS and incubated with primary antibody at 37°C for 1 hour or at 4°C overnight, followed by Alexa Fluor 488 (goat anti rabbit) for 30 minutes at room temperature. Stained cells were examined using Zeiss fluorescence microscope.

Invasion assay and activity of MMP-2/9 analysis

Cells were cultivated to 80% confluence on the 12-well plates. Then, we observed the procedures of cellular growth at 24 h. All the experi-

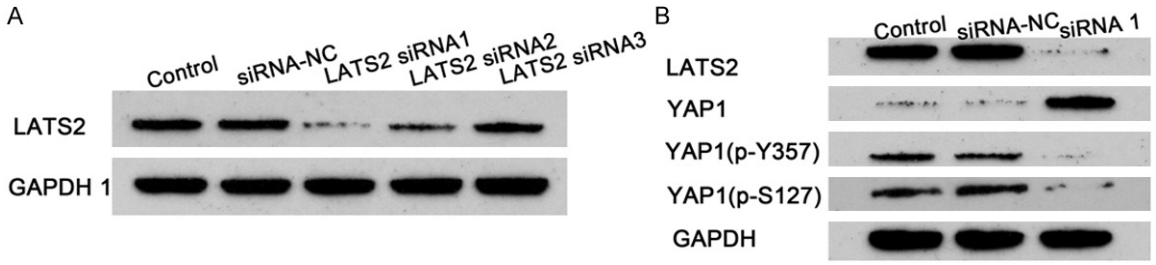

Figure 2. The effects of LATS2 on the expression level of YAP1. A. Western blot analysis examined four siRNAs to target human LATS2 protein. B. Western blot analysis revealed the effects of LATS2 on the expression level of YAP1.

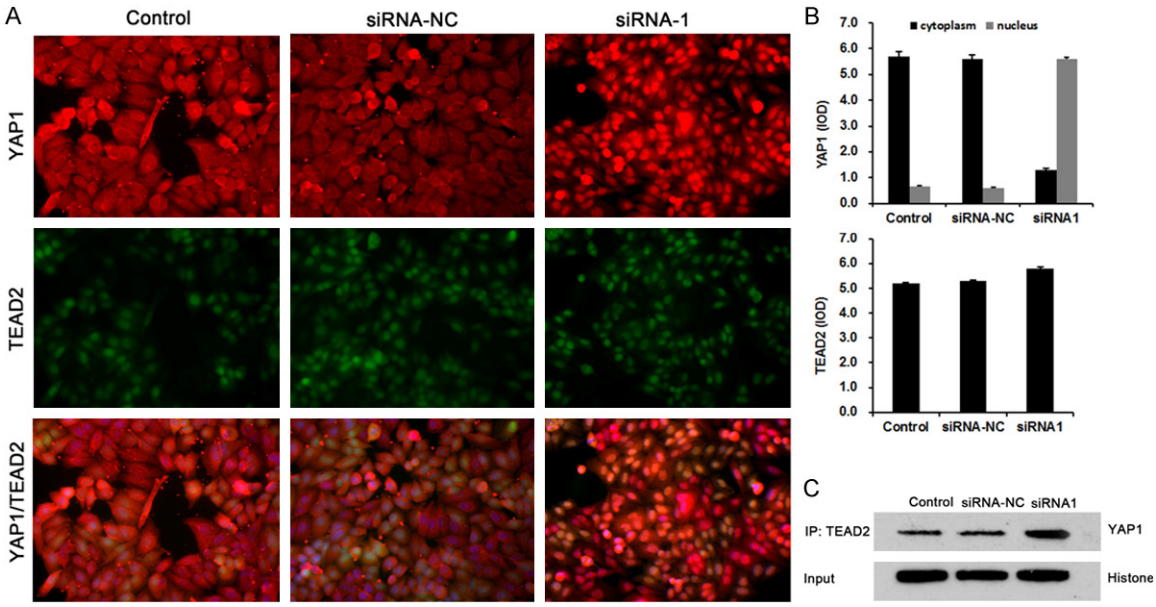

Figure 3. A and B. Subcellular localization of endogenous YAP and TEAD2 after knock-down of LATS2 by siRNA. Immunofluorescent staining of YAP1 and TEAD2 in HepG2 cells transfected with siRNA1 (SIRT1) or scramble control siRNA. C. HepG2 cells were transfected with siRNA1 (LATS2). Lysates were immunoprecipitated with anti-TEAD2 and immunoblotted with anti-YAP1 antibody. LATS2 knockdown impaired the YAP1/TEAD2 association.

ments were repeated in triplicate. The transwell invasion chambers were used to evaluate cell invasion. Then cells invading across the membrane were counted under a light microscope.

Assays MMP-2/9 activity was detected by gelatin zymography as described previously [19].

Statistical analysis

Each experiment was repeated at least three times. Data were shown as mean ± s.d and analyzed using SPSS 18.0. Statistical comparisons between groups were analyzed using Student's t-test and a two-tailed $P < 0.05$ was considered to indicate statistical significance.

Results

Expression of LATS2, YAP1 and TEAD2 in human HCC cell lines

To better understand the role of LATS2 in HCCs, we first measured the expression of LATS1 in human hepatocellular carcinoma cell lines. We found that LATS1 protein levels in 7 HCC cell lines (Bel-7402, HepG2, Hep3B, SMMC7721, Huh7, 97H and 97L cell lines) were significantly lower than that of the human embryo liver L02 cell line (**Figure 1**). However, LATS2 was low expressed in HCC cell lines (Hep3B, SMMC-7721, Huh7, 97H and 97L cell lines) and high expressed in HCC cell lines (Bel-7402 and HepG2) and human embryo liver L02 cell line.

YAP1 was highly expressed in in HCC cell lines (Hep3B, SMMC7721, Huh7, 97H and 97L cell lines) and lowly expressed in HCC cell lines (Bel-7402 and HepG2) and human embryo liver L02 cell line. This result indicated that LATS2 could be the negative regulator of YAP1 in HCCs. Moreover, the expression tendency of TEAD2 was similar to that of YAP1 in HCC cell lines and human embryo liver cell line. Thus, LATS2 and HepG2 were used for further experiments.

LATS2 phosphorylates YAP in HCC

The HepG2 cells were transfected with LATS2 siRNAs and with negative control siRNA containing scrambled random sequence, respectively. We examined four siRNAs to target human LATS2 as described above. As shown in **Figure 2A**, siRNA-1 effectively blocked the expression of TMSG1 mRNA. Western blot analysis revealed dramatic reduction of 89.2% with siRNA-1, 62.4% with siRNA-2, and 5.2% with siRNA-3 in the levels of LATS2 protein after transfection of siRNA in HepG2 cells, compared with LATS2 negative control siRNA or the control (untransfected) group ($P < 0.05$). Thus, the cells transfected with LATS2 shRNA1 were used for further experiments.

As YAP is the downstream effector of Hippo signaling pathway and has been found to be critical for HCC tumorigenesis, we decided to detect the effect of LATS2 on the expression level of YAP1. The levels of LATS2-dependent phosphorylation of YAP1 were measured using phospho-specific antibodies that recognize the LATS2 phosphorylation site S127 and Y357 on YAP1 [20, 21]. As shown in **Figure 2B**, the expression levels of YAP1 (p-Y357) and YAP1 (p-S127) in siRNA1 group were much lower than control group and siRNA-NC group, indicating that LATS2 inhibition caused a major decrease in YAP1 phosphorylation.

YAP cytoplasmic localization, and transactivating activity are modulated by LATS2

YAP activity is known to depend on its cellular localization which, in turn, is determined by its phosphorylation status. Phosphorylation of YAP at Ser127 induces translocation of YAP from the nucleus to cytoplasm, thus reducing the transcriptional activity of TEAD [18]. Based on these, we examined the localization of YAP in the cells treated with LATS2 siRNA1. Consistent with the decreased phosphorylation level of YAP, siRNA1 treatment induced translocation of YAP from the cytoplasm to nucleus as visualized by immunofluorescent staining, whereas siRNA1 treatment did not affect the expression of TEAD2 in nucleus (**Figure 3A** and **3B**). Next, we demonstrated whether LATS2 inhibition could affect the association between YAP1 and TEAD2 proteins. HepG2 cells were infected with LATS2 siRNA1. Lysates were immunoprecipitated with anti-TEAD2 and immunoblotted with anti-YAP1 antibody. Consistent with immunofluorescent staining results, LATS2 inhibition dramatically increased the interaction of YAP1 with TEAD2 (**Figure 3C**). These results clearly demonstrated that LATS2 inhibition-mediated YAP1 -dephosphorylation significantly increased YAP1 nuclear accumulation, which lead to increase the interaction of YAP1 with TEAD2.

LATS2 siRNA increased cell invasion and activity of MMP-2/9 in HCCs

The Yes-associated protein (YAP) is a downstream effector of the Hippo signaling pathway and acts as transcriptional co-activators of TEAD and regulate transcription of target genes involved in cell growth, proliferation and survival [22, 23]. Based on these, we next examined the effect of LATS2 on invasion of HCC. As shown in **Figure 4A**, at 24 h, the cell invasion capacity in siRNA1 group was similar with control group and siRNA-NC group, but at 48 h and 72 h, the cell invasion was significantly increased in siRNA1 group compared with control group and siRNA-NC group. Moreover, we determined the activities of MMP-2/9, which are closed related to cancer invasion and metastasis [24]. The supernatant of cultured cells was collected and the gelatinase activity was assayed with gelatin zymography. As shown in **Figure 4B**, LATS2 siRNA did not affect the MMP-2 and MMP-9 activity at 24 h. However, the activity of MMP-2 and MMP-9 was apparently increased at 48 h and 72 h in the supernatant of the LATS2 siRNA cells compared with the siRNA-NC cells or control cells, which indicated that the downregulation of LATS2 expression increased the activity of MMP-2 and MMP-9 protein. In conclusion, LATS2 inhibition increased invasion capacity and MMP-2/9 activity in a time-dependent manner.

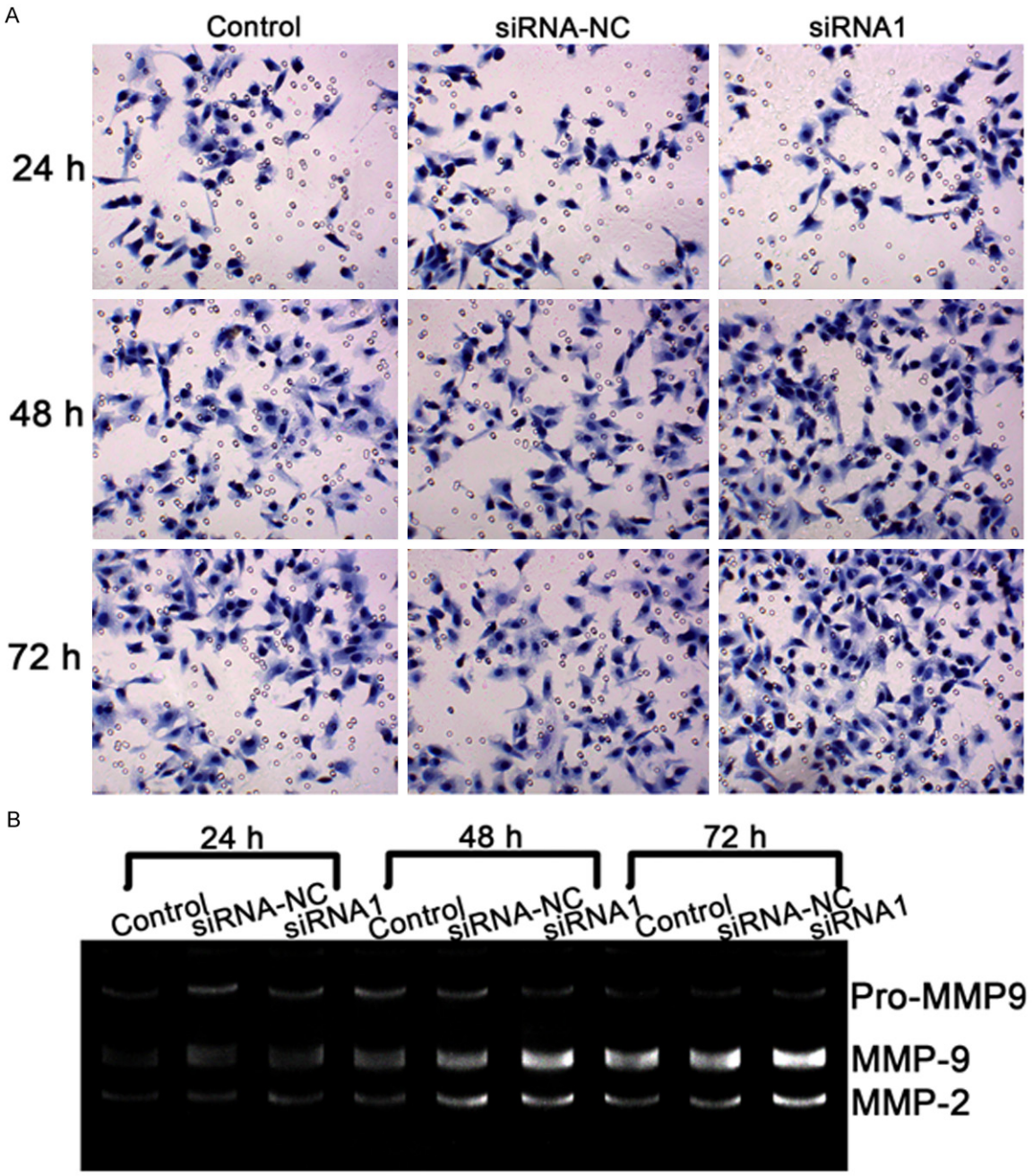

Figure 4. The effects of LATS2 siRNA on cell invasion and activity of MMP-2/9 in HCCs. A. Transwell assays showed that the LATS2 siRNA transfected cells displayed dramatically increased invasion ability compared with the untreated cells or Negative Control siRNA transfected cells. B. The activity of MMP-2/9 was examined by Gelatin zymography. The activities of MMP-2 and MMP-9 in LATS2 siRNA group were obviously higher than that in the siRNA-NC or control group.

Discussion

Many oncogenes are found to be regulated by LATS2, which raises the possibility that LATS2 acts as a tumor suppressors [25, 26]. In this study, we identified that LATS2 inhibition attenuated YAP phosphorylation and significantly increased YAP1 nuclear accumulation and in turn the upregulation of the YAP1/TEAD2 association, leading to YAP1/TEAD2 transcriptional activation and cell invasion in HCC cells. Our findings revealed the regulatory mechanism of

YAP1 by the LATS2-mediated phosphorylation that was involved in liver tumorigenesis.

LATS encodes a putative Ser/Thr protein kinase and is the central player in the Hippo signaling pathway. Previous reports suggested that LATS2 involved in the regulation of the cell cycle and overexpression resulted in cell cycle arrest at the G1/S and G2/M phase via inhibiting CDK2 activity and Cdc2-Cyclin B kinase activity, respectively [27, 28]. Moreover, Dysregulation of LATS2 expression has been reported to involve in tumorigenesis by regulating cell proliferation, cell growth and apoptosis in various human cancers including breast, ovary and liver cancers [28-30]. In this study, we first measured the expression of LATS2 in 7 human hepatocellular carcinoma cell lines. The western blot analysis showed that LATS2 was low expressed in HCC cell lines (Hep3B, SMMC7721, Huh7, 97H and 97L cell lines) and high expressed in HCC cell lines (Bel-7402 and HepG2) and human embryo liver L02 cell line.

YAP, as a transcriptional coactivator, is the downstream effector of the pathway. YAP functions are regulated by multiple post-translational modifications, including phosphorylation, SUMOylation, acetylation, and methylation [11, 31-34]. The phosphorylation levels of YAP at S127 and Y357 play a critical role for the translocation of YAP from the nucleus to cytoplasm. Wang P, et al. revealed that PP1A dephosphorylated YAP1 at serine 127 and dissociated it from 14-3-3 binding, thus leading to its nuclear retention and transcriptional activation [35]. Zhang H, et al. showed that abeta [25-35] induced YAP translocation from cytoplasm to nucleus accompanied with the increased phosphorylation on Y357 [20]. In this study, we detected the effect of LATS2 on the phosphorylation levels of YAP1-S127 and YAP1-Y357. Western blot analysis demonstrated that LATS2 inhibition caused a major decrease in both YAP1-S127 and YAP1-Y357 phosphorylation. Consistent with previous work, immunofluorescent staining revealed that inhibition of LATS2 attenuated YAP1 phosphorylation, which in turn induced nuclear translocation of YAP. As a transcriptional coactivator, Several structural studies have provided valuable insight into the details of YAP signaling via complexes with partner proteins [36]. The TEAD proteins is the major partners of YAP and YAP/TEAD complex is necessary for growth promoting activity of YAP oncogene [37]. In this study, the inhibition of LATS2 dramatically increased the interaction of YAP1 with TEAD2, indicating that LATS2 could attenuate YAP1/TEAD2 by upregulating YAP1 phosphorylation levels. In particular, several lines of evidence indicate YAP as an oncogene in several human cancers [38, 39]. In human cancers, YAP1 is often overexpressed and promotes proliferation and tumor growth [40]. In mouse liver, transgenic overexpression of YAP causes enlargement of this organ and the eventual development of hepatic tumors [10, 41]. YAP phosphorylation promotes YAP cytoplasmic retention and degradation, resulting in inhibition of cell motility, proliferation and oncogenesis [42, 43]. Consistent with these studies, LATS2 inhibition significantly increased the invasion capacity and MMP2/9 activities, indicating that LATS2-mediated YAP1 phosphorylation could suppress tumor metastasis.

In conclusion, our results indicate that LATS2 inhibition suppresses YAP1 phosphorylation and promotes nuclear localization of YAP1 and the YAP1/TEAD2 interactions, which in turn induces HCC progression. The identification of LATS2 affecting YAP1 phosphorylation and the delineation of their roles in the control of YAP activity may represent a promising target for anticancer therapies.

Disclosure of conflict of interest

None.

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