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Knockdown of the Hippo transducer YAP reduces proliferation and promotes apoptosis in the Jurkat leukemia cell

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Abstract. Leukemia and lymphoma are common hematological malignancies in children and young adults, which pose a tremendous threat to the survival of these young patients worldwide, despite availability of various effective treatments. The Hippo pathway is a novel-signaling pathway that regulates organ size, cell proliferation, apoptosis and tumorigenesis. The chief component of this pathway is the transducer yes-associated protein (YAP) which is over-expressed in numerous categories of tumors. However, little is known about the effect of YAP in hematological malignancies. In the present study, YAP expression was screened in several leukemia and lymphoma cell lines, and high YAP expression was demonstrated in Jurkat cells. To further unravel its effect on the biological behavior of Jurkat cells, lentivirus transduced short hairpin RNA (shRNA) technique was used to silence YAP. As expected, the YAP-specific shRNA dramatically inhibited YAP expression at the mRNA and protein levels. Reduced leukemia cell proliferation and increased cell apoptosis were demonstrated in YAP knockdown Jurkat cells. It was also demonstrated that YAP knockdown resulted in deregulated expression of a cluster of downstream genes crucial to cell proliferation or apoptosis, including protein kinase B, B-cell lymphoma 2 (BCL2) and BCL2 like protein 1. Consequently, the results of the present study established that suppression of YAP expression serves an important role in Jurkat cell proliferation and apoptosis, which may serve as a potential therapeutic target.

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Introduction

Hematological malignancies, represented by leukemia and lymphoma, are life-threating diseases associated with a poor prognosis. Although treatment outcome of the patients has improved tremendously in the era of combination chemotherapy, autologous or allogeneic hematopoietic stem cell transplantation, a significant proportion of patients remain refractory to or relapse following treatment and ultimately succumb to the disease. Therefore, numerous strategies have been developed to treat such diseases (1-3). In the past three decades, target-based therapies have established milestones in the treatment of leukemia. Two typical successful examples are the applications of all-trans retinoic acid in acute promyelocytic leukemia (APL) and tyrosine kinase inhibitor in chronic myeloid leukemia (CML) (4,5). These impressive achievements are inspired by the understanding of the precise mechanisms of these two types of leukemia. There is no doubt that further research into the pathogenesis of leukemia leading to novel therapeutic targets will benefit patients enormously (6).

The Hippo signaling pathway is an important modulator involved in the regulatory process of cell proliferation, differentiation and death. It was first identified in the course of identifying tumor suppressor genes in Drosophila, which serves a vital role in controlling organ growth or regeneration (7,8). The Hippo signaling pathway consists of three interactive elements: The upstream sensor molecules, downstream transcriptional components and core kinase parts. The core components of Hippo pathway in mammals include mammalian STE20-Like1 (MST1)/(MST2), protein salvador homolog 1, large tumor suppressor (LATS1/LATS2), MOB1/MOB2, yes-associated protein (YAP) and Tafazzin (TAZ) (9). Classical cascade signaling activation of the Hippo pathway begins from the phosphorylation and activation of MST1/MST2 by upstream molecules. Then, the phosphorylated MST1/2 further activate LATS1 and LATS2 under the help of MOB kinase activator (MOBKL)1, MOBKL2, which also experiences activation by MST1/2 (10). Finally, the activated LATS1/2 phosphorylates the transcriptional co-activator YAP and TAZ, binding to 14.3.3 proteins in the cytoplasm, which accelerates the sequestration and degradation of YAP/TAZ (11,12).

Abnormal expression of YAP has been identified in various human tumors, including renal cell carcinoma, pancreatic

cancer, breast cancer, cholangiocarcinoma and medulloblastoma (13-17). Several groups have documented the aberrant expression or genetic abnormalities of the Hippo pathway in hematological malignancies, especially in acute leukemia and lymphoproliferative neoplasms (18-22). In the present study, YAP expression was screened in several leukemia and lymphoma cell lines, and demonstrated high YAP expression in Jurkat cells. To elucidate its effect on cell biology of Jurkat cells, lentivirus mediated short hairpin RNA (shRNA) technique that targets the silencing of YAP was performed. As anticipated, YAP expression was significantly suppressed at the mRNA and protein levels by the YAP-specific shRNA. Notably, decreased leukemia cell proliferation and enhanced cell apoptosis were demonstrated in YAP knockdown Jurkat cell line. Taken together, these results indicate that YAP may be a potential novel therapeutic target for certain types of leukemia.

Materials and methods

Materials. MTT was purchased from Beyotime Institute of Biotechnology (Shanghai, China); Dulbecco's modified Eagle's medium (DMEM) was obtained from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA); Fetal bovine serum (FBS) was purchased from Hyclone (GE Healthcare Life Sciences, Logan, UT, USA); interleukin (IL)-3, IL-6 and stem cell factor (SCF) were purchased from R&D Systems, Inc., (Minneapolis, MN, USA); TRIzol reagent and Lipofect 2000 were purchased from Invitrogen (Thermo Fisher Scientific, Inc.); SYBR Green PCR Master Mix was obtained from Takara Bio, Inc., (Otsu, Japan); pLenti6.3/V5-DEST lentivirus vector and Packaging Mix were obtained from Invitrogen (Thermo Fisher Scientific, Inc.); RIPA protein lysis buffer was obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany); Antibodies for western blotting including anti-YAP, protein kinase B (AKT1), B-cell lymphoma 2 (BCL2) and BCL2 like protein 1 were bought from Santa Cruz Biotechnology, Inc., (Dallas, TX, USA); apoptosis analysis kits (7-AAD, Annexin V-FITC) were obtained from Nanjing KeyGEN Biotech Co., Ltd., (Nanjing, China) and the ECL-PLUS/kit was obtained from GE Healthcare (Chicago, IL, USA).

Cell lines and culture. Jurkat, Daudi, SuDHL-4, NB4, HL60 and 293 cells were obtained from Shanghai Institute of Hematology (Shanghai, China); Cultured in DMEM supplemented with 10% heat-inactivated FBS, 100 U/ml of penicillin and 100 μg/ml of streptomycin. Hematopoietic stem cells and progenitor cells (HSC/HPC) cells were collected from normal murine bone marrow mononuclear cells (MNC). Briefly, mice were sacrificed and the bone marrow was extracted under sterile conditions from femurs and tibias. The bone marrow suspension was filtered through a 40-μm-cell strainer and centrifuged for 5 min at 500 x g. Bone marrow mononuclear cells were washed in DMEM supplemented with 10% FBS after lysis of red blood cells, centrifuged, resuspended and cultured in the above medium supplemented with IL-3 (10 ng/ml), IL-6 (10 ng/ml) and SCF (50 ng/ml) cytokine cocktail. Ethical approval was given for the present study by ethics committee of Xinhua Hospital (Shanghai, China). All cells were incubated in a humidified atmosphere containing 5% CO_2 at 37˚C.

Cell transduction. shRNA loaded by lentivirus was used in the present study. YAP targeting shRNA was synthesized and purchased from NOVOBIO (Shanghai Novobio, Co., Ltd., Shanghai, China; http://www.novobiosci.com). shRNA specific sequences for YAP located number 1,522 nt of YAP mRNA (PL/shRNA-YAP-1522): 5'-CACCGACCAATAGCT CAGATCCTTTCGAAAAAGGATCTGAGCTATTGGTC-3'; negative control sequences (NC): 5'-TTCTCCGAACGTGTC ACGT-3'. Jurkat cells were cultured in 24-well plates. Cells were transduced by the lentiviral supernatant harboring the NC or shRNA-YAP packaged in 293 cells, with 1 μg/ml polybrene (Shanghai GenePharma Co., Ltd., Shanghai, China) added to enhance transduction efficacy. Following co-culture for 24 h, the medium was replenished. Fluorescence density was observed under a fluorescence microscope.

Cell proliferation test. Cell proliferation was measured by MTT assay. Cells were seeded in 96-well-plates at a density of $1x10^4$ cells/well in 100 μl DMEM medium, cultured in a CO_2 incubator for 24-72 h, washed and replenished with 100 μl fresh medium. A total of 10 μl MTT stock solution was added to each well and further incubated at 37˚C for 4 h in a CO_2 incubator. This was followed by adding 100 μl the SDS-HCl solution to each well and further incubation at 37˚C for 4 h in a CO_2 incubator. Each sample was mixed by pipetting up and down. Absorbance was read at 570 nm. Cell proliferation rate at 24, 48 and 72 h were calculated.

RNA isolation and reverse transcription quantitative polymerase chain reaction (RT-qPCR). In order to quantitatively analyze mRNA expression profile, RT-qPCR was performed. Total cell RNA was extracted with TRIzol reagent according to manufacturer's protocol. Reverse transcription was done with M-MLV at 25˚C for 5 min, 42˚C for 60 min and 72˚C for 5 min. cDNA amplification was managed by SYBR-Green PCR Master Mix kit according to the manufacturer's protocol. Merlin, protein salvador homolog 1 (Sav1), MST1/2, LATS1/2 and YAP genes of normal murine HSC/HPC were amplified by different primers. Human YAP, AKT, BCL2, TP53, BCL2L1, tumor necrosis factor receptor superfamily member 6 (FAS), caspase 8 (CASP8), FAS associated death domain (FADD), CASP3, BAX, tumor necrosis factor receptor type 1-associated DEATH domain (TRADD) protein genes were separately amplified using specific artificially synthesized primers, with the homo-actin house-keeping gene serving as internal control. Sequences of the primers used in the study are presented in Table I. Expression values were analyzed using the comparative Cq ($2^{-\Delta\Delta Cq}$) (23).

Western blot analysis. Protein was extracted using RIPA lysis buffer according to protocol and protein concentration was quantified by bicinchoninic acid protein assay kit. A total of 50 μg of protein was loaded in 10% SDS-PAGE and electrophoresed, then transferred to polyvinylidene difluoride membrane, which was further blocked and incubated with 5% defatted milk powder for 2 h at room temperature. This was then incubated with a specific primary antibody

Table I. Primer sequences used in the present study.

A, Mouse

Number	Gene	Primer	Primer sequence (5'-3')
1	Merlin	IF	CGGACACTGGGGCTTCGGGAAACCT
		IR	TCCAAAATCTGCTTCTTCACCTGTA
2	SAV1	IF	GACTGGACAATGAGAGGGAGAAAAT
		IR	TGATGACTCTACTCGTTCCCAGCCA
3	MST1	IF	CAGAGTCAGGGCGGGAGTGTCAACG
		IR	AATAGTTGTCTTTCAGATCTTTGTC
4	MST2	IF	TAACAGATACAATGGCAAAACGCAA
		IR	AATGTTGGTGGTGGGTTTGTAGGGA
5	LATS1	IF	ATGTGGTTTATCGTTCTGAAAGCCC
		IR	ATGAGGGGGGAGGGGGAGTGGTGCC
6	LATS2	IF	CTGCCACAACTTACTCTGGAAATAG
		IR	TTGATAAGGTCCAAACTTCGGGGTG
7	YAP1	IF	CCTTCTTCAAGCCGCCTGAGCCCAA
		IR	AGGGATCTCAAAGGAGGACTGCCGG

B, Human

Number	Gene	Primer	Primer sequence (5'-3')
1	YAP	IF	TAGCCCTGCGTAGCCAGTTA
		IR	TCATGCTTAGTCCACTGTCTGT
2	AKT1	IF	GTCATCGAACGCACCTTCCAT
		IR	AGCTTCAGGTACTCAAACTCGT
3	BCL2	IF	GGTGGGGTCATGTGTGTGG
		IR	CGGTTCAGGTACTCAGTCATCC
4	TP53	IF	CAGCACATGACGGAGGTTGT
		IR	TCATCCAAATACTCCACACGC
5	BAX	IF	CCCGAGAGGTCTTTTTCCGAG
		IR	CCAGCCCATGATGGTTCTGAT
6	BCL2L1	IF	GAGCTGGTGGTTGACTTTCTC
		IR	TCCATCTCCGATTCAGTCCCT
7	TRADD	IF	GCTGTTTGAGTTGCATCCTAGC
		IR	CCGCACTTCAGATTTCGCA
8	FAS	IF	TCTGGTTCTTACGTCTGTTGC
		IR	CTGTGCAGTCCCTAGCTTTCC
9	CASP8	IF	GTGGGGTAATGACAATCTCGG
		IR	TCAAAGGTCGTGGTCAAAGC
10	FADD	IF	GCTGGCTCGTCAGCTCAAA
		IR	ACTGTTGCGTTCTCCTTCTCT
11	CASP3	IF	GAAATTGTGGAATTGATGCGTGA
		IR	CTACAACGATCCCCTCTGAAAAA
12	Homo-actin	IF	AGCGAGCATCCCCCAAAGTT
		IR	GGGCACGAAGGCTCATCATT

SAV1, protein salvador homolog 1; MST1, mammalian STE20-Like1; MST2, mammalian STE20-Like2; LATS1, large tumor suppressor homolog 1; LATS2, large tumor suppressor homolog 2; AKT1, protein kinase B; YAP, yes-associated protein; BCL2, B cell lymphoma 2; BCL2L1, BCL2 like protein 1; TRADD, Tumor necrosis factor receptor type 1-associated DEATH domain protein; FAS, tumor necrosis factor receptor superfamily member 6; FADD, FAS associated death domain; CASP, caspase; IR, reverse primer; IF, forwards primer.

(YAP, SC376830; BCL2, SC7382 and BCL2L1, SC56021 all 1:100 dilution; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) in TBS with 0.05% Tween 20 overnight at 4˚C or room temperature for 4 h, washed to remove primary antibody and further incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (goat anti-mouse IgG-HRP; SC2005; 1:2,000; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. ECL Chemiluminescence Detection kit and X-ray film were used for HRP-conjugated secondary antibody. The Bio-Rad Gel Imaging System was used to analyze signals. Densitometry was performed using ImageJ version 1.8.0 software (National Institutes of Health, Bethesda, MD, USA).

Cell cycle analysis. For experiments on cell cycle analysis, cells were harvested and resuspended in fresh DMEM medium, centrifuged (250 x g for 5 min at 4˚C) and washed with PBS and fixed with cold 70% ethanol added drop wise to the pellet while vortexing. Following fixing for 30 min at 4˚C and washing with PBS, the fixed cells were stained with 7-AAD (DNA dye) in the presence of RNase for 30 min at room temperature in the dark room. The samples were then analyzed on a FACsort flow cytometer using CellQuest 1.0 software (BD Biosciences, Franklin Lakes, NJ, USA).

Cell apoptosis analysis. In cell apoptosis experiment, cells were harvested and suspended in fresh DMEM medium, then resuspended in Annexin-binding buffer following centrifugation (300 x g, 5 min, 4˚C) and washed according to the corresponding protocols. Annexin V-FITC and 7-AAD working solution (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) were added to the cells and incubated in the dark at room temperature for 15 min. Following incubation, Annexin-binding buffer was added and the samples were kept on ice to be analyzed as soon as possible by flow cytometry. Cell apoptosis was measured and analyzed by CellQuest 1.0 software (BD Biosciences, Franklin Lakes, NJ, USA).

Statistical analysis. Values were expressed as the mean ± standard deviation. Statistical analysis was performed using SPSS software version 13.0 (SPSS, Inc., Chicago, IL, USA). Quantitative data was analyzed by one-way analysis of variance, multiple comparisons between different groups was performed using S-N-K method. $P<0.05$ was considered to indicate a statistically significance difference. Each experiment was repeated at least three times.

Results

YAP is highly expressed in Jurkat cells. Previously the expression level of several vital components of the Hippo signal transduction pathway has been studied and it was demonstrated that Merlin, Sav1, MST1/2, LATS1/2 and YAP were all expressed in normal murine HSC/HPC (Fig. 1A). This provided the basis for the hypothesis that the Hippo signaling pathway may serve certain important roles in murine HSC/HPC function. Notably, the Hippo signaling pathway did not influence the proliferation and colony formation abilities when YAP was ectopically overexpressed in HSC/HPC cells (data not shown). Therefore hematological malignant cells were focused on in the present study. YAP expression levels were screened in several leukemia and lymphoma cell lines including Jurkat, Daudi, SuDHL-4, NB4 and HL60. Considering that components of the Hippo pathway were expressed in normal human HSC/HPC, normal human HSC/HPCs were used as a control. Compared with the control, it was demonstrated that YAP is highly expressed in Jurkat cells at the mRNA and protein levels (Fig. 1B-D), Daudi cells also had a relatively high expression level of YAP while SuDHL-4 had lower expression level (Fig. 1B-D). NB4 and HL-60 had expression level comparable to the control (data not shown).

YAP knockdown in Jurkat cells. As YAP was highly expressed in Jurkat cells, the function of YAP in Jurkat cells was further investigated, utilizing YAP-shRNA to knockdown YAP expression in Jurkat cells. Lentivirus harboring YAP-shRNA exhibited a high titer ($5.2x10^8$ TU/ml) following successful packaging. The lentivirus titration was measured by calculating multiplicity of infection through sequential dilution (data not shown). Jurkat cells were successfully transduced by lentivirus and Jurkat cells were observed using a fluorescence microscope system or light microscope following transduction (Fig. 2A and B). RT-qPCR results demonstrated that mRNA expression of YAP was dramatically decreased in the YAP knockdown (YAP-shRNA) group compared with the NC group, demonstrating 75% interfering efficacy ($P<0.01$, Fig. 2C). Correspondingly, the western blotting experiment further demonstrated that YAP protein expression was markedly reduced in these cells (Fig. 2D). All these data demonstrated that YAP was successfully silenced by lentivirus mediated shRNA interference.

Influence of YAP silencing on proliferation of Jurkat cells. Uncontrolled cellular proliferation is the hallmark of leukemia cells. To check whether knockdown of YAP by shRNA elicited a suppressive effect on Jurkat cell proliferation, an MTT assay was utilized to investigate the effect of YAP silencing. Compared with the NC counterpart, the proliferation rate of YAP-shRNA Jurkat cells was affected in a time-dependent manner and this was significant following 3 days ($P<0.05$; Fig. 3A). Furthermore, the cell cycle pattern was analyzed by flow cytometry (FCM). FCM demonstrated that knockdown of YAP in Jurkat cells boosted the percentage of G_0/G_1 phase cells from 43.6-50.8% (Fig. 3B-C). Finally, the downstream genes associated with cell proliferation or cell cycle regulation including AKT1 and P53 were investigated. It was demonstrated that AKT1 gene expression, which is crucial to the PI3K-AKT proliferation pathway, was decreased in YAP-shRNA transduced Jurkat cells compared with the NC group (Fig. 3D). These data provided strong evidence that YAP silencing could inhibit Jurkat cell proliferation through G_0/G_1 arrest.

Effect of YAP knockdown on apoptosis of Jurkat cells. Deregulation of apoptosis is another hallmark of leukemia cells. To investigate the effect of YAP knockdown on Jurkat cell apoptosis. FCM analysis was performed on YAP knockdown Jurkat cells following 7-AAD and Annexin V-FITC double staining. Compared with the NC group, YAP knockdown significantly promoted early apoptosis (8.05% vs. 1.93%)

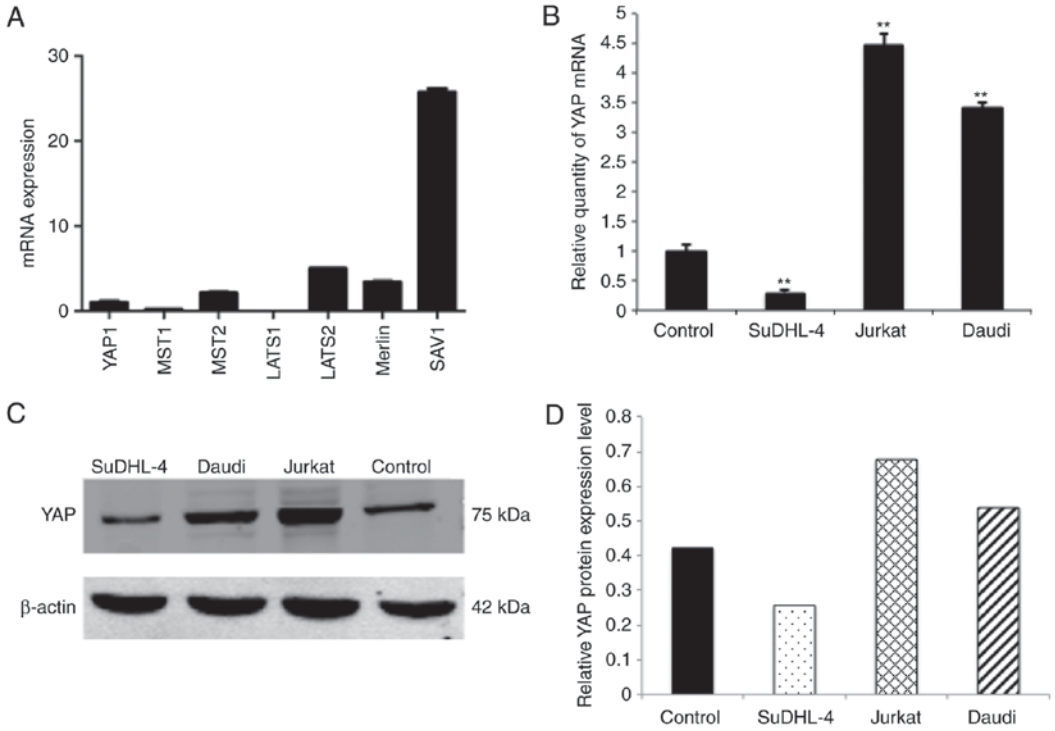

Figure 1. Hippo core kinase components expression profiles. (A) mRNA expression of Hippo core kinase components in normal murine HSC/HPC. (B) mRNA expression level of YAP gene in lymphoma and leukemia cell lines. **P<0.01 vs. the control. Normal human HSC/HPC was used as control. (C) Western blotting analysis of YAP protein expression in lymphoma and leukemia cell lines. Normal human HSC/HPC was used as control. (D) Densitometry analyses of YAP protein expression in lymphoma and leukemia cell lines. Normal human HSC/HPC was used as control. YAP, yes associated protein; sh, short hairpin; LATS large tumor suppressor; MST, mammalian STE20-Like; SAV1, protein salvador homolog 1; HSC/HPC, hematopoietic stem cells and progenitor cells.

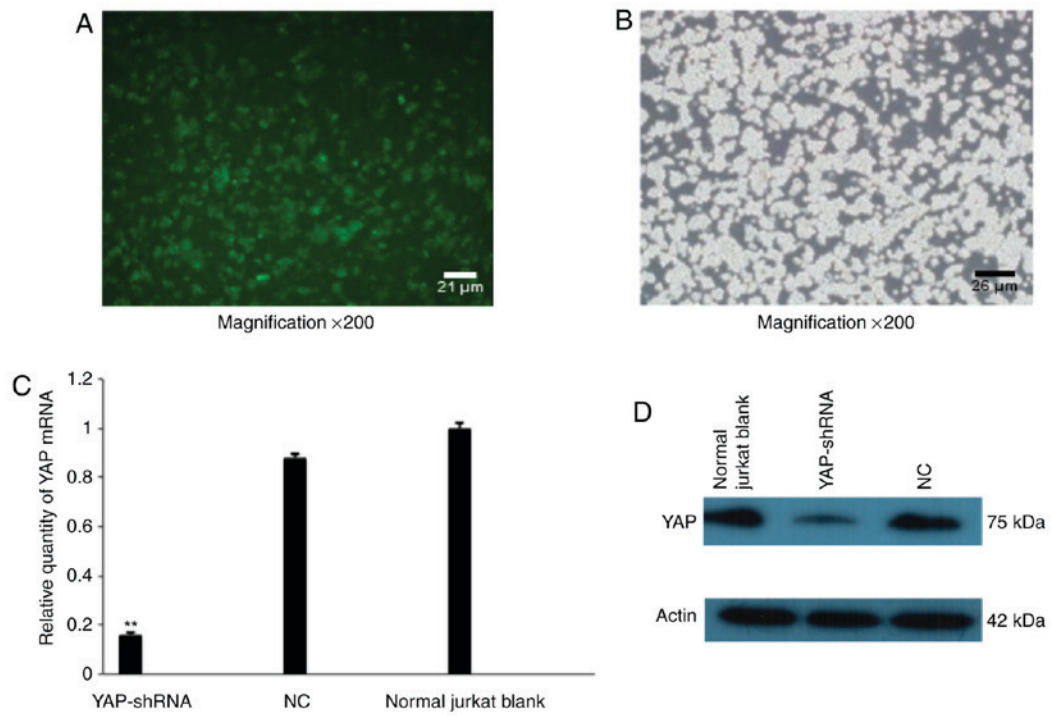

Figure 2. Lentivirus-mediated YAP knockdown in Jurkat cells. (A) Fluorescent microscopy demonstrating knockdown of YAP in Jurkat cell by transduction with YAP-shRNA (magnification, x200). (B) Light microscopy demonstrating knock down of YAP in Jurkat cell by transduction with YAP-shRNA. (C) mRNA level of YAP as measured by reverse transcription-polymerase chain reaction. Data are expressed as the mean ± standard deviation. **P<0.01 vs. the NC group. (D) Protein expression of YAP as detected by western blotting. sh, short hairpin; YAP, yes associated protein; NC, negative control.

and late apoptosis rate (27.8% vs. 9.9%) in Jurkat cells (P<0.01; Fig. 4A-C). Furthermore, typical morphological features of apoptosis were also discerned in YAP knockdown Jurkat cells (data not shown). The expression levels of several downstream

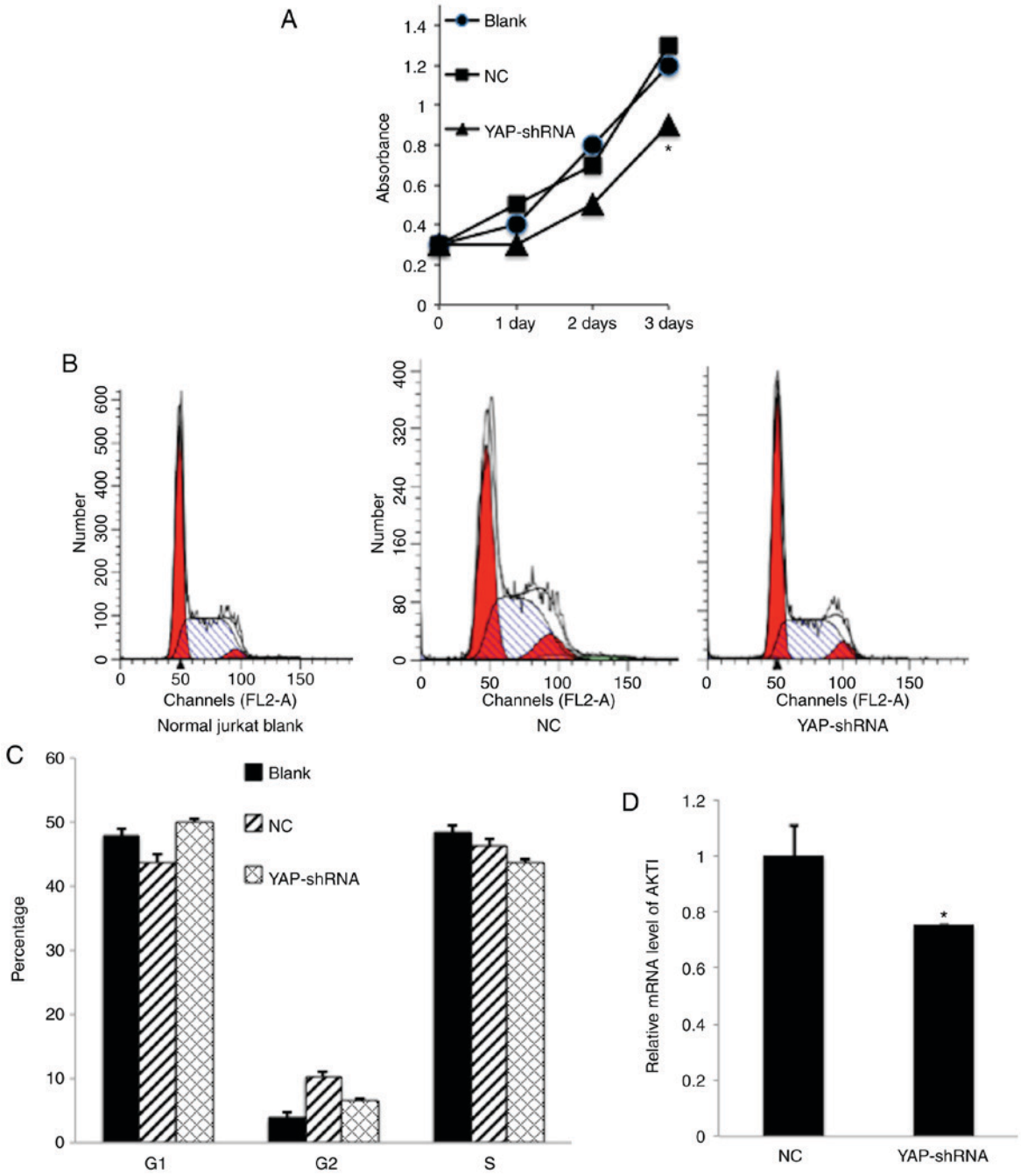

Figure 3. YAP knockdown inhibits proliferation of Jurkat cells. (A) Cells proliferation as detected by MTT assay. (B) Cell cycle distribution as measured by flow cytometry. (C) Cell cycle distribution as represented by bar chart. (D) Quantitative analysis by measuring the relative mRNA expression levels of AKT1 to β-actin. Data are expressed as the mean ± standard deviation. *P<0.05 YAP-shRNA vs. NC group. AKT, protein kinase B; YAP, yes associated protein; sh, short hairpin; NC, negative control.

apoptosis or anti-apoptosis associated genes including BCL2, FAS, CASP8, FADD, CASP3, BCL2L1, BAX and TRADD were further verified. It was demonstrated that expression levels of BCL-2 and BCL2L1 genes associated with anti-apoptosis were significantly reduced (P<0.05; Fig. 4D and F), While no significant difference was observed in the mRNA expression level of FAS, CASP8, FADD, CASP3, BAX and TRADD between YAP knockdown Jurkat cells and the NC group (Fig. 4E). All these results imply that YAP silencing induced apoptosis in Jurkat cells through deregulating the expression of anti-apoptosis associated proteins.

Discussion

Despite rapid development in drugs including targeted therapies, hematological malignancies are still challenging diseases to treat due to high incidence of chemo-refractoriness and relapse. There is an urgent need to investigate novel pathogenesis mechanisms and develop more novel therapies. The Hippo signaling pathway contains a large group of interactive proteins, which participate in regulating cell proliferation, differentiation and apoptosis. Importantly, deregulation of Hippo pathway correlates with pro-oncogenic or anti-oncogenic processes. Function of Hippo

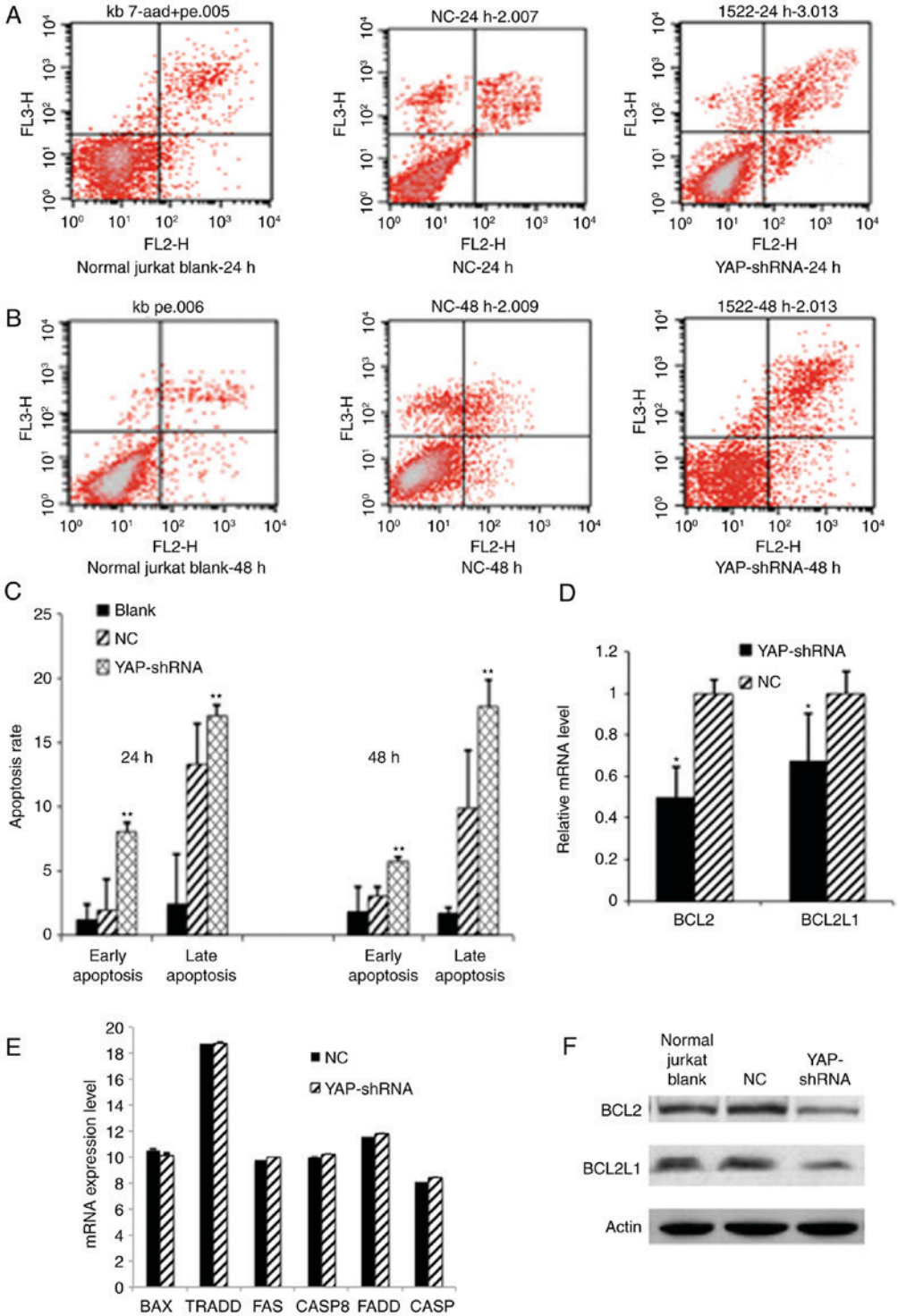

Figure 4. YAP knockdown induced apoptosis of Jurkat cells. (A) Apoptosis was analyzed by FCM using double staining with FITC-labeled Annexin V and 7-AAD at 24 h. Cells undergoing early apoptosis are Annexin V-FITC+/7-AAD-(lower right quadrant), whereas cells undergoing late apoptosis are Annexin V-FITC+/7-AAD+ (upper right quadrant). (B) Apoptosis analyzed by FCM at 48 h. (C) Apoptosis as represented by a bar chart. Quantitative data was analyzed by one-way analysis of variance and multiple comparisons between different groups were performed using the Student-Newman-Keuls method (**P<0.01 YAP-shRNA vs. NC group). (D) Quantitative analysis by measuring the relative mRNA expression levels of BCL2 or BCL2L1 to β-actin. Data are expressed as the mean ± standard deviation. *P<0.05 vs. the NC group. (E) Other pro-apoptotic or anti-apoptotic associated genes expression profile. (F) Western blotting analysis of BCL2 or BCL2L1 protein expression. FITC, fluorescein isothiocyanate; YAP, yes associated protein; FCM, flow cytometry; BCL2, B cell lymphoma 2; BCL2L1, BCL2 lie protein 1; CASP, caspase; TRADD, tumor necrosis factor receptor type 1-associated DEATH domain; FAS, tumor necrosis factor receptor superfamily member 6; FADD, FAS associated death domain; NC, negative control; sh, short hairpin.

pathway has been clarified in a number of organs or tissues, but its function in the hematopoietic system is still unknown. It is therefore interesting to investigate Hippo pathway in normal hematopoiesis and hematological malignancies.

It is controversial whether the Hippo pathway is involved in the hematological system. Several study groups have documented the deregulated expression profile or epigenetic alterations of Hippo pathway in hematological malignancies,

especially in acute leukemia or lymphoproliferative neoplasms (24,25). In acute myeloid leukemia (AML), overexpression of the LATS2 gene was detected in 32 *de novo* AML patients, suggesting that LATS2 may be associated with leukemogenesis (18). Another group reported that AML patients with t (8;12) translocation generates the MST2-ETV6 fusion gene, which is a potential oncogene (19). In acute lymphoblastic leukemia (ALL) patients harboring t (12;21) chromosome translocation, a vital upstream component in the Hippo pathway named KIBBRA is demonstrated to be highly methylated and is the main underlying leukemogenesis event in this specific subtype of leukemia (20). The LATS2 gene is also a methylation modification target hotspot in ALL patients; its lower expression due to methylation predicts patients' poor prognosis (21). In addition, in adult T cell or natural killer leukemia/lymphoma, decreased expression of LATS2 gives rise to lower expression of proapoptotic genes or causes chemotherapy resistance (26). The Hippo pathway abnormality is also seen in APL pathogenesis, because YAP is required for APL transcriptional activation (27,28). In mantle cell lymphoma, decreased expression of MOBKL2 and LATS2, important component proteins of the Hippo pathway, has been detected in certain patients and indicates poor outcome (22). MST1 deficiency is highly predisposed to develop T-cell ALL under mutagenic stimulation. Furthermore, $MST1^{(-/-)}$ mice display rapid formation of lymphoma in a p53 knockout model and obvious chromosomal instability has been demonstrated in $MST1^{(-/-)}$ lymphocytes (29). YAP is overexpressed in CML cells. Knockdown of YAP by siRNA or inhibiting the function of YAP using verteporfin (VP) not only inhibits the proliferation, induces the apoptosis of CML cells but also reduces the expression of YAP target genes c-myc and survivin (30).

On the contrary, the ectopic overexpression of YAP in the hematopoietic system, reported by another research group, does not influence HSC function neither during steady state nor in situations of hematopoietic stress (31). The author speculates that the discrepant nature of the different tissues may explain the mechanism of cell over-proliferation or excessive growth in solid tissues, while not in the hematopoietic system. Machado-Neto *et al* (32) demonstrated that YAP may not correlate with hematological malignancies including AML, ALL or myelodysplastic syndromes.

In the authors' previous study, in order to investigate whether Hippo pathway is associated with haematopoiesis, the expression level of several vital components of the Hippo signal transduction pathways in normal mice was checked, and demonstrated that Merlin, Sav1, MST1/2, LATS1/2 and YAP were all expressed in normal murine HSC/HPC. It was therefore hypothesized that Hippo signal pathway may serve certain important roles in murine HSC/HPC function. Notably, it did not influence proliferation and colony formation abilities when ectopic YAP was overexpressed in HSC/HPC cells, which is consistent with what has been reported by other groups (31). In the present study, hematological malignancies were the main focus. YAP expression levels were screened in several leukemia and lymphoma cell lines and it was demonstrated that YAP was highly expressed in Jurkat cells at the mRNA and protein levels. Considering that YAP has oncogeneic features, YAP was silenced by shRNA in Jurkat cells and the consequences were investigated. Growth arrest, inhibition of cell proliferation, apoptosis, and differentiation are the most well-characterized effects of tumor suppressor response, therefore we tested for these aspects of Jurkat cells following silencing YAP.

An MTT assay was conducted to assess cell metabolic activity associated with cell proliferation. It was demonstrated that Jurkat cell proliferation was weakened in YAP knockdown group, compared with the control group. Furthermore, G_0/G_1 phase cells were increased in YAP knockdown group compared with the control group. Finally, several downstream genes associated with cell proliferation or cell cycle regulation were checked. It is well known that inhibition of the PI3K-AKT pathway could inhibit proliferation of tumor cells. In the present study the expression levels of AKT1 were demonstrated to be decreased in the YAP knockdown group, compared with the control group. Therefore, the results indicated that cell proliferation was inhibited in Jurkat cells following silencing YAP by interfering with the PI3K-AKT pathway.

FCM was also used to analyze apoptosis in the YAP-knockdown Jurkat cells. It was demonstrated that YAP knockdown remarkably increased early and late apoptosis in Jurkat cells. In addition, morphological alterations of apoptotic Jurkat cells including nuclear fragmentation or condensation could be easily seen in the YAP silenced group, but not in the NC or normal untreated group. Finally, the expression level of several downstream genes associated with cell apoptosis was measured, and decreased levels of Bcl-2 and BCL2L1 genes were observed, the two of have anti-apoptotic properties. These results suggested that knockdown of YAP by shRNA enhanced apoptosis through deregulating the expression levels of pro-apoptotic or anti-apoptotic associated genes.

Cottini *et al* (33) demonstrated a tumor suppressor function for YAP in multiple myeloma, lymphoma and leukemia cell lines. In an experimental model, damaged DNA activates apoptosis by a p53-independent pathway in a process guided by nuclear re-localization of the ABL1 kinase and its interaction with YAP, demonstrating that low YAP1 levels prevent nuclear ABL1-induced apoptosis in these hematological malignancies, supporting the hypothesis that YAP1 is involved in the tumor suppressor process. In contrast, the present study demonstrated that Jurkat cells express a high level of YAP1. Furthermore, knockdown of the YAP1 in the Jurkat cell caused growth arrest and apoptosis, indicating that YAP1 promotes oncogenesis process. Possible explanations for the discordance between these two different studies were analyzed: i) The work of Cottini *et al* (33) has demonstrated that Jurkat cells express a low level of YAP protein, while the cells of the present study expressed a relatively high level. This dissimilarity may originate from different cellular metabolic conditions, excessive passage, mutation or other clonal evolution. ii) Indeed, the YAP protein possesses dual functions, like a double-edged sword, as a pro-oncogenic or anti-oncogenic molecule. Its double role seems to be dependent on its phosphorylation pattern: Phosphorylation by LATS1/2 at Ser127 promotes YAP sequestration in the cytoplasm and prevents its interaction with TEAD transcription factors, while phosphorylation by c-Abelson murine leukemia viral oncogene (ABL) at Tyr357 upon DNA damage confers a tumor suppressive function to YAP (25,33). Furthermore, It was also demonstrated that YAP

expression was high in Daudi cells and for the next study YAP will be knocked down in Daudi cells to investigate what kind of role YAP serves in these cells.

In conclusion, the current study initially revealed a high expression level of YAP in Jurkat cells. Lentivirus mediated shRNA decreased YAP expression in Jurkat cells. Silencing of YAP expression by shRNA suppressed the growth and promoted apoptosis of Jurkat cells. These results demonstrated that YAP serves an important role in leukemogenesis, which may be a potential target for the treatment of leukemia in the future.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LM made substantial contributions to the design of the present study. RW and HY performed the experiments, data collection and data analysis. RW and LM wrote the manuscript. JW, XD, LC provided help in conceiving and designing the study. SH made substantial contributions in data analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Ethical approval was given for the present study by ethics committee of Xinhua hospital.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Döhner H, Weisdorf DJ and Bloomfield CD: Acute myeloid leukemia. N Engl J Med 373: 1136-1152, 2015.
2. Terwilliger T and Abdul-Hay M: Acute lymphoblastic leukemia: A comprehensive review and 2017 update. Blood Cancer J 7: e577, 2017.
3. Shi Y: Current status and progress of lymphoma management in China. Int J Hematol 107: 405-412, 2018.
4. de Thé H, Pandolfi PP and Chen Z: Acute promyelocytic leukemia: A paradigm for oncoprotein-targeted cure. Cancer Cell 32: 552-560, 2017.
5. Saglio G, Cilloni D, Rancati F and Boano L: Glivec and CML: A lucky date. J Biol Regul Homeost Agents 18: 246-251, 2004.
6. Rashidi A, Weisdorf DJ and Bejanyan N: Treatment of relapsed/refractory acute myeloid leukaemia in adults. Br J Haematol 181: 27-37, 2018.
7. Wu S, Huang J, Dong J and Pan D: Hippo encodes a Ste-20 family protein kinase that restricts cell proliferation and promotes apoptosis in conjunction with salvador and warts. Cell 114: 445-456, 2003.
8. Harvey K and Tapon N: The Salvador-Warts-Hippo pathway-an emerging tumour-suppressor network. Nat Rev Cancer 7: 182-191, 2007.
9. Hong W and Guan KL: The YAP and TAZ transcription co-activators: Key downstream effectors of the mammalian Hippo pathway. Semin Cell Dev Biol 23: 785-793, 2012.
10. Sansores-Garcia L, Atkins M, Moya IM, Shahmoradgoli M, Tao C, Mills GB and Halder G: Mask is required for the activity of the Hippo pathway effector Yki/YAP. Curr Biol 23: 229-235, 2013.
11. Couzens AL, Knight JD, Kean MJ, Teo G, Weiss A, Dunham WH, Lin ZY, Bagshaw RD, Sicheri F, Pawson T, *et al*: Protein interaction network of the mammalian Hippo pathway reveals mechanisms of kinase-phosphatase interactions. Sci Signal 6: rs15, 2013.
12. Zhao B, Li L, Lei Q and Guan KL: The Hippo-YAP pathway in organ size control and tumorigenesis: An updated version. Genes Dev 24: 862-874, 2010.
13. Fernandez-L A, Squatrito M, Northcott P, Awan A, Holland EC, Taylor MD, Nahlé Z and Kenney AM: Oncogenic YAP promotes radio resistance and genomic instability in medulloblastoma through IGF2-mediated Akt activation. Oncogene 31: 1923-1937, 2012.
14. Cao JJ, Zhao XM, Wang DL, Chen KH, Sheng X, Li WB, Li MC, Liu WJ and He J: YAP is overexpressed in clear cell renal cell carcinoma and its knockdown reduces cell proliferation and induces cell cycle arrest and apoptosis. Oncol Rep 32: 1594-1600, 2014.
15. Li X, Liu Y, Zhang C, Niu Q, Wang H, Che C, Xie M, Zhou B, Xu Y, Zhang Q, *et al*: Stiehopusjaponieus acidic mucopolysaccharide inhibits the proliferation of pancreatic cancer SW1990 cells through Hippo-YAP pathway. Oncotarget 8: 16356-16366, 2017.
16. Kim HM, Jung WH and Koo JS: Expression of Yes-associated protein (YAP) in metastatic breast cancer. Int J Clin Exp Pathol 8: 11248-11257, 2015.
17. Pei T, Li Y, Wang J, Wang H, Liang Y, Shi H, Sun B, Yin D, Sun J, Song R, *et al*: YAP is a critical oncogene in human cholangiocarcinoma. Oncotarget 6: 17206-17220, 2015.
18. Gholami M, Mirfakhraie R, Movafagh A, Jalaeekhoo H, Kalahroodi R, Zare-Abdollahi D and Zare-Karizi S: The expression analysis of LATS2 gene in de novo AML patients. Med Oncol 31: 961, 2014.
19. Ogawa S, Yokoyama Y, Suzukawa K, Nanmoku T, Kurita N, Seki M, Maie K, Suyama T, Takaiwa N, Sakata-Yanagimoto M, *et al*: Identification of a fusion gene composed of a Hippo pathway gene MST2 and a common translocation partner ETV6 in a recurrent translocation t(8;12)(q22;p13) in acute myeloid leukemia. Ann Hematol 94: 1431-1433, 2015.
20. Hill VK, Dunwell TL, Catchpoole D, Krex D, Brini AT, Griffiths M, Craddock C, Maher ER and Latif F: Frequent epigenetic inactivation of KIBRA, an upstream member of the Salvador/Warts/Hippo (SWH) tumor suppressor network, is associated with specific genetic event in B-cell acute lymphocytic leukemia. Epigenetics 6: 326-332, 2011.
21. Jiménez-Velasco A, Román-Gómez J, Agirre X, Barrios M, Navarro G, Vázquez I, Prósper F, Torres A and Heiniger A: Down regulation of the large tumor suppressor 2(LATS2/KPM) gene is associated with poor prognosis in acute lymphoblastic leukemia. Leukemia 19: 2347-2350, 2005.
22. Hartmann EM, Campo E, Wright G, Lenz G, Salaverria I, Jares P, Xiao W, Braziel RM, Rimsza LM, Chan WC, *et al*: Pathway discovery in mantle cell lymphoma by integrated analysis of high-resolution gene expression and copy number profiling. Blood 116: 953-961, 2010.
23. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
24. Garcia-Souza LF and Oliveira MF: Mitochondria: Biological roles in platelet physiology and pathology. Int J Biochem Cell Biol 50: 156-160, 2014.

25. Aqeilan RI: Hippo signaling: To die or not to die. Cell Death Differ 20: 1287-1288, 2013.
26. Kawahara M, Hori T, Chonabayashi K, Oka T, Sudol M and Uchiyama T: Kpm/Lats2 is linked to chemosensitivity of leukemic cells through the stabilization of p73. Blood 112: 3856-3866, 2008.
27. Lapi E, Di Agostino S, Donzelli S, Gal H, Domany E, Rechavi G, Pandolfi PP, Givol D, Strano S, Lu X and Blandino G: PML, YAP, and p73 are components of aproapoptotic autoregulatory feedback loop. Mol Cell 32: 803-814, 2008.
28. Strano S, Fausti F, Di Agostino S, Sudol M and Blandino G: PML Surfs into HIPPO tumor suppressor pathway. Front Oncol 3: 36, 2013.
29. Kim TS, Lee DH, Kim SK, Shin SY, Seo EJ and Lim DS: Mammalian sterile 20-likekinase 1 suppresses lymphoma development by promoting faithful chromosome segregation. Cancer Res 72: 5386-5395, 2012.
30. Li H, Huang Z, Gao M, Huang N, Luo Z, Shen H, Wang X, Wang T, Hu J and Feng W: Inhibition of YAP suppresses CML cell proliferation and enhances efficacy of imatinib in vitro and in vivo. J Exp Clin Cancer Res 35: 134, 2016.
31. Jansson L and Larsson J: Normal hematopoietic stem cell function in mice with enforced expression of the Hippo signaling effector YAP1. PLoS One 7: e32013, 2012.
32. Machado-Neto JA, de Melo Campos P, Olalla Saad ST and Traina F: YAP1 expression in myelodysplastic syndromes and acute leukemias. Leuk Lymphoma 55: 2413-2415, 2014.
33. Cottini F, Hideshima T, Xu C, Sattler M, Dori M, Agnelli L, Ten Hacken E, Bertilaccio MT, Antonini E, Neri A, *et al*: Rescue of Hippo coactivator YAP1 triggers DNA damage-induced apoptosis in hematological cancers. Nat Med 20: 599-606, 2014.