Yes-associated protein expression is associated with poor prognosis in patients with colorectal cancer

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Abstract. Colorectal cancer (CRC) is the third leading cause of cancer-related death worldwide. The aim of the present study was to investigate the expression of yes-associated protein (YAP) in CRC tissues, and to determine the relationship between the expression levels of YAP and the clinicopathological characteristics and prognosis of patients with CRC. Bioinformatics analysis was conducted to examine the expression of YAP and its correlation with clinicopathological characteristics and key genes, using functional enrichment analysis. Immunohistochemistry was used to detect YAP expression in 181 CRC tissue samples and 30 normal colorectal mucosa samples. Western blotting and reverse transcription-quantitative PCR were performed to detect the expression of YAP and β-catenin in CRC cells, and cellular proliferation was assessed using a Cell Counting Kit-8 assay. Finally, apoptosis was analyzed using flow cytometry. Immunohistochemical staining indicated that the positive expression rate of YAP in CRC tissues was 73.5%, which was significantly higher than that in normal colorectal mucosa samples. The expression of YAP in CRC was associated with histological differentiation, lymph node metastasis and Duke's stage. However, no significant associations were observed between YAP expression and age, sex and T stage. Downregulation of YAP promoted the proliferation and the inhibited apoptosis of CRC cells, and YAP expression was positively correlated with that of β-catenin in both CRC tissues and cells. Furthermore, YAP expression was upregulated in CRC tissues, which was correlated with tumor progression and prognosis. Therefore, YAP expression may be used as an independent predictor of poor prognosis in patients with CRC, and the underling molecular mechanism may be associated with the combined effect of Hippo and Wnt/β-catenin signaling.

Introduction

Colorectal cancer (CRC) is the third most common malignancy worldwide, accounting for 9% of cancer-associated deaths in both men and women in the United States (1). In 2020, the total number of new cases of CRC reported in the United States was 147,910, with 53,200 deaths, the latter roughly one-third of the former. The CRC morbidity rate is highest in the >65-year age group (exceeding 50%), with a mortality rate of ~70%. Notably, the incidence of CRC among individuals aged <50 years has now reached 12%. With respect to tumor location, the incidence of colon cancer is 3.5 times greater than the incidence of rectal cancer (2). In 2020, data from The Global Cancer Observatory revealed that between 2014 and 2018, the incidence of CRC increased at an annual rate of ~8% (3). Although the prognosis of CRC has improved greatly in recent years, with advances in medicine and technology, cancer relapse, drug resistance, metastasis and other clinical features still result in high mortality in patients with CRC (4,5). Therefore, an improved understanding of the molecular mechanisms underlying CRC carcinogenesis and progression is essential for the development of specific markers and effective therapeutic strategies for patients with CRC.

Yes-associated protein 1 (YAP) is a transcriptional coactivator and regulator of the Hippo signaling pathway (6). The *YAP* gene is located on human chromosome 11q22 and is a candidate oncogene (7). As a transcription coactivator, YAP can negatively regulate the Hippo signaling pathway, which is involved in the regulation of organ size by modulating cellular polarity, proliferation and apoptosis (8). When the Hippo signaling pathway is activated, YAP is phosphorylated and accumulates in the cytoplasm by binding to the 14-3-3 protein, which inhibits downstream target gene transcription (9). Conversely, when the Hippo signaling pathway is blocked or inactivated, YAP cannot be phosphorylated. Instead, it is transported to the nucleus, where it interacts with the sequence-specific transcription factor TEAD, and other transcription factors, to promote the transcription and expression

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of target genes associated with cellular proliferation, such as baculoviral inhibitor of apoptosis repeat-containing 5 (BIRC5)/survivin (10). This causes cells to proliferate uncontrollably, resulting in tumorigenesis. Hence, YAP expression is upregulated in various cancer types, including lung (11), gastric (12), ovarian (13), colorectal (7), and breast cancer (14).

The Hippo signaling pathway plays a crucial role in cellular differentiation, proliferation, apoptosis and tumorigenesis (15). Previous studies have shown that the Hippo signaling pathway acts as a tumor suppressor in multiple tumor types, including breast cancer (16), oral squamous cell carcinoma (17), hepatocellular carcinoma (18), and ovarian cancer (19). Furthermore, the Hippo signaling pathway negatively regulates YAP (20), whose expression is an important prognostic factor in CRC (8). As a transcriptional coactivator, YAP can induce the expression of various negative regulators of apoptosis, including members of the inhibitor of apoptosis protein family, such as BIRC5/survivin (21). The Hippo/YAP and Wnt/β-catenin signaling pathways interact with each other to maintain cellular stability, and are associated with the apoptosis and proliferation of CRC cells (22). Moreover, β-catenin is an important transcriptional coactivator that regulates downstream targets in the Wnt/β-catenin pathway that are involved in cellular differentiation, proliferation and apoptosis (23). BIRC5/survivin is a target gene of the Hippo/YAP and Wnt/β-catenin signaling pathways. The aim of the present study was to investigate the role and molecular mechanism of YAP in the occurrence and development of CRC.

Materials and methods

Patients and tissue samples. A total of 181 consecutive patients with newly diagnosed, pathologically confirmed, CRC were recruited from the Department of Pathology at the First Affiliated Hospital of Anhui Medical University (Hefei, China), between November 2007 and December 2013. The study was approved by the Ethics Committee of Anhui Medical University, and written informed consent was obtained from each participant. None of the patients had received preoperative chemotherapy or radiotherapy. The cohort included 112 men and 69 women, of which the median age was 57.5 years (range, 31-89); 44, 105 and 32 patients had well, moderately and poorly differentiated adenocarcinomas, respectively; 82 patients showed evidence of lymph node metastasis on pathological examination. Furthermore, 26 patients presented with Duke's stage A, 53 Duke's stage B, and 102 Duke's stage C-D disease.

A total of 30 normal colorectal mucosa samples were taken at a distance of >5.0 cm from the tumor margin. All specimens were fixed with 10% neutral formalin at 25°C for 24 h, embedded in paraffin, cut into 4.0-μm-thick serial sections, and used for immunohistochemistry. Complete clinical data were available for all patients. Follow-up information was obtained by telephone or from outpatient records. Complete follow-up data were available for 82 patients (patients who had died due to unsuccessful treatment before the time point were considered as having complete follow-up data).

Cell culture and transfection. RKO (ATCC® CRL-2577™) and LoVo (ATCC® CCL-229™) cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), and were maintained in 6-well plates [2×10^5 cells per 2.0 ml culture medium; 90% of DMEM supplemented with 10% fetal bovine serum (both HyClone; Cytiva)] in a humidified incubator at 37°C (5% CO_2). Once the cells had reached 30-50% confluence, the siRNA-Lipofectamine mixture was prepared per the following steps: i) 5.0 μl Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific Inc.) was diluted in 250 μl Opti-MEM/well, gently aspirated 3-5 times, and then incubated at 37°C for 5 min; ii) 5.0 μl siRNA (100 nM) was diluted in 250 μl Opti-MEM/well (Invitrogen; Thermo Fisher Scientific, Inc.; final concentration, 50 nM); iii) Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) was incubated for 10 min, mixed with the siRNA, and allowed to stand at 25°C for 20 min; iv) the siRNA-Lip mixture was added to the cells and transfection medium to a total volume of 2.0 ml; v) the plate was gently shaken until thoroughly mixed, and incubated at 37°C (5% CO_2) for 6 h; and vi) the medium was replaced with 2.0 ml fresh culture medium, and incubated for a further 24 h. The siRNA sequences were as follows: YAP1 sense, 5'-GGUGAUACUAUCAACCAAATT-3'; and antisense, 5'-UUUGGUUGAUAGUAUCACCTT-3'; scramble siRNA control sense, 5'-UUCUCCGAACGUGUCACGUTT-3'; and antisense, 5'-ACGUGACACGUUCGGAGAATT-3'.

Immunohistochemistry. Briefly, sections were deparaffinized and rehydrated in a descending alcohol series (100, 90, 75 and 50%). Endogenous peroxidase was blocked with 0.3% hydrogen peroxide. The sections were boiled in sodium citrate buffer (0.01 M, pH 6.0) for 15 min (120°C, 0.097 MPa) for antigen retrieval. After cooling to room temperature for 30 min, the sections were blocked with 5% normal goat serum at 25°C for 1 h, followed by incubation with a YAP1 antibody (1:100; cat. no. ab205270; Abcam) in blocking buffer at 4°C overnight. The next day, the sections were incubated with Goat Anti-Rabbit IgG H&L (HRP) secondary antibody (1:5,000; cat. no. ab7090; Abcam) at 25°C for 30 min. The slides were developed with 3,3-diaminobenzidine solution at 25°C for 5 min, and counterstained with hematoxylin. The total YAP immunostaining score was obtained through multiplying staining power by positive cell percentage. The score standard for staining intensity was based on different degrees of tissue staining without specific background staining: No visible staining, 0; light yellow, 1; tan, 2; and dun, 3. The score standard for positive cell evaluation criteria was as follows: Positive cells ≤5%, 0; >5% but ≤25%, 1; >25% but ≤50%, 2; >50% but ≤75%, 3; and >75%, 4.

RNA extraction and reverse transcription-quantitative (RT-q) PCR. Total RNA was extracted from cell samples using RNAiso Plus reagent (Takara Bio, Inc.). Reverse transcription (RT) was performed using PrimeScript RT-polymerase (Takara Bio, Inc.), The RT procedure comprised 42°C for 2 min for gNDA eraser and 37°C for 15 min for 6 cycles, followed by 85°C for 5 sec. qPCR was performed to amplify the cDNA templates using the SYBR Premix Ex Taq II kit (Takara Bio, Inc.). The qPCR thermocycling conditions were as follows: 95°C for 10 sec during the hold stage, followed by 95°C for 5 sec and 60°C for 30 sec for 40 cycles. β-actin was used as the reference gene, and each sample was run in triplicate. Data

were normalized to the expression of β-actin, and relative expression was calculated using the $2^{-\Delta\Delta Cq}$ method (24). The primer sequences were as follows: YAP forward, 5'-TGGGAC TCAAAATCCAGTGTC-3' and reverse, 5'-CCATCTCCT TCCAGTGTTCC-3'; and β-actin, forward 5'-GGCATCCAC GAAACTACCTT-3' and reverse, 5'-CGGACTCGTCATACT CCTGCT-3'.

Western blot analysis. Total protein was extracted from whole-cell lysates using RIPA buffer (Invitrogen; Thermo Fisher Scientific Inc.) containing protease inhibitors, and protein concentration was determined by BCA analysis (Thermo Fisher Scientific Inc.). A 30-μg sample of protein per lane was separated by denaturing 8-10% SDS-PAGE and transferred PVDF membrane (EMD Millipore). The membranes were blocked with 5% skim milk in PBST at room temperature for 1 h, and then incubated with an anti-YAP1 antibody (1:1,000; cat. no. ab205270; Abcam) at 4°C overnight. The next day, the membranes were washed three times with PBST (PBS containing 0.1% Tween-20) for 5 min each, and then incubated with rabbit anti-sheep IgG H&L (HRP) (1:10,000; cat. no. ab6747; Abcam) at 25°C for 1 h. Enhanced chemiluminescence reagents (Thermo Fisher Scientific, Inc.) were used to detect the immunoreactive bands. Each experiment was performed in triplicate and repeated three times. The gray values of the blots were determined using ImageJ software version 1.47 (National Institutes of Health) for statistical analysis.

Cell Counting Kit-8 assay. Cells transfected with scramble control and YAP-siRNA were incubated in 96-well plates (10,000 cells per plate, 5 wells per group) in 100 μl medium. The medium was replaced with fresh culture medium daily. After 0, 24, 48, 72 and 96 h, 10 μl CCK-8 (Invitrogen; Thermo Fisher Scientific, Inc.) was added to the appropriate wells, and the plate was incubated at 37°C for 1 h. The MTT formazan crystals were solubilized with 100 μl dimethyl sulfoxide (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C for 4 h. The optical density (OD) at a wavelength of 570 nm was measured using a microplate reader (Thermo Fisher Scientific, Inc.). Cell proliferation inhibition rate was calculated as follows: [(control group OD value-experimental group A value)/control group OD value] x 100%. The experiment was repeated three times, and cell proliferation curves were constructed using Prism version 8.0 (GraphPad Software, Inc.).

Flow cytometry. The apoptotic cells were detected using a FACSCalibur flow cytometer (BD Biosciences) using FlowJo software version 10 (FlowJo LLC). The cells were digested with 0.25% trypsin-EDTA. The Annexin V-FITC Apoptosis Detection Kit (Invitrogen; Thermo Fisher Scientific, Inc.) was used to detect apoptotic cells induced by 10 mmol/l 5-fluorouracil (5-FU; Sigma-Aldrich) for 24 h at 37°C (5% CO_2). Cells were washed twice with precooled phosphate-buffered saline and the cell concentration was adjusted to 5×10^5/ml. The cells were then resuspended in 400 μl 1X Annexin V. Annexin V-FITC staining solution (5.0 μl) was added at 4°C for 15 min, followed by 10 μl propidium iodide staining solution, and incubated at 2-8°C for 5 min. The experiment was repeated three times.

Bioinformatics analysis. Gene Expression Profiling Interactive Analysis (GEPIA) was used to mine the expression and gene regulation network of YAP in CRC. LinkedOmics was used to identify differential gene expression associated with YAP, and to analyze Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. Moreover, colon adenocarcinoma and rectum adenocarcinoma data were downloaded from The Cancer Genome Atlas for further evaluation of YAP expression in a wider cohort of patients with colorectal cancer, according to different clinical and pathological features; however, no other significant association was found (data not shown), thus only data from GEPIA were include in the current study.

Statistical analysis. The Pearson's χ^2 test was used to determine the association between the expression levels of YAP and the clinicopathological characteristics of patients with CRC. A two-tailed unpaired t-test was used to compare the differences between two groups. One-way ANOVA followed by Tukey's multiple comparison test was used to compare cellular proliferation ability between different cell types. Survival curves were plotted using the Kaplan-Meier method and compared using the log-rank test. All statistical analyses were performed using SPSS v23.0 (IBM Corp.), and P<0.05 was considered to indicate a statistically significant difference.

Results

Upregulation of YAP in CRC tissues. The expression profile of YAP protein is as follows: Cytoplasmic and nuclear staining of YAP was positive in well-differentiated CRC tissues (Fig. 1A); diffuse nuclear expression of YAP was positive in moderately differentiated CRC tissues (Fig. 1B); cytoplasmic and nuclear staining of YAP was positive in moderate to poorly differentiated CRC tissues (Fig. 1C); and nuclear expression of YAP was positive in poorly differentiated CRC tissues (Fig. 1D). The positive expression rate of YAP was 73.5% (n=133/181), with a low expression rate of 25.9% (n=47/181) and a high expression rate of 47.5% (n=86/181). Among the 30 normal colorectal mucosa samples, only five stained positive for YAP with low plasma expression (16.7%). No nuclear or nuclear plasma staining was detected. The positive expression rate of YAP was significantly higher in CRC tissues (n=133/181) than in normal colorectal mucosa samples (16.7%) (P<0.0001). These findings were consistent with the results of bioinformatics analysis (Fig. 2A).

Upregulation of YAP is correlated with the aggressive characteristics of CRC. The expression of YAP in CRC was found to correlate with histological differentiation, lymph node metastasis and Duke's stage (P<0.05). However, it was not correlated with other clinicopathological characteristics of CRC, such as age, sex and T stage (Table I). Furthermore, YAP was highly expressed in both the cytoplasm and nucleus, as well as nuclear plasma of CRC tissues. Nuclear and nuclear plasma-positivity were associated with lymph node metastasis and Duke's stage (P<0.05). No significant correlations with other clinicopathological characteristics were reported. Cytoplasmic positivity was not significantly correlated with any of the clinicopathological characteristics analyzed in the present study (Table II). In addition, bioinformatics analysis revealed that the relative expression of the *YAP* gene was positively
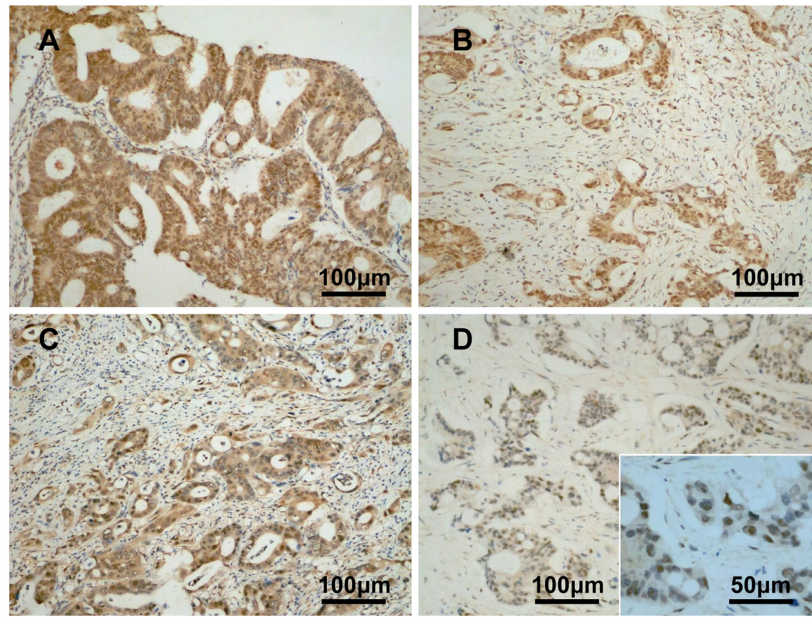
Figure 1. Expression profile of YAP protein in CRC tissues. (A) Cytoplasmic and nuclear staining of YAP was positive in well-differentiated CRC tissues. (B) Diffuse nuclear expression of YAP was positive in moderately differentiated CRC tissues. (C) Cytoplasmic and nuclear staining of YAP was positive in moderate to poorly differentiated CRC tissues. (D) Nuclear expression of YAP was positive in poorly differentiated CRC tissues. YAP, yes-associated protein; CRC, colorectal cancer.

correlated with M stage (P<0.05; Wilcoxon test), N stage (P<0.05; Kruskal-Wallis Test) and pathological stage (P<0.05; Kruskal-Wallis Test). Furthermore, the expression level of the *YAP* gene was higher in patients with microsatellite stable (MSS)-CRC than in those with microsatellite instability-high (MSI-H)-CRC (P<0.0001; Wilcoxon test) (Fig. 2B).

YAP-knockdown cell line construction. RKO and LoVo cells were transfected with YAP-siRNA and scramble control, and the expression levels of YAP were determined by western blotting and RT-qPCR 48 h after transfection. Western blot analysis showed that compared with the normal and scramble control groups, YAP protein expression was markedly reduced in the YAP-siRNA group 48 h after transfection. Furthermore, the PCR results showed that compared with the normal and scramble control groups, RKO and LoVo cell YAP DNA levels were significantly reduced in the YAP-siRNA group, which was equivalent to the 5-FU control group (Fig. 2C).

Association between YAP and the proliferation of CRC cells. RKO and LoVo cell proliferation were detected 24 h after transfection using the Cell Counting Kit-8 assay at 0-, 24-, 48-, 72- and 96-h time points. Cell viability is shown as a proliferation curve based on the OD value at 450 nm. Compared with the scramble control groups, the proliferation rates of the YAP-siRNA RKO (P<0.001) and YAP-siRNA LoVo (P<0.0001) groups were significantly decreased, and were similar to those of the 5-FU groups (P<0.05; Fig. 2D).

Association between YAP and CRC cell apoptosis. In the present study, apoptosis was represented as the late plus the early apoptotic rate. The apoptotic rates of RKO and LoVo cells were stained by Annexin V/PI and detected by flow cytometry 48 h after transfection. The results showed that compared with

Table I. Association between YAP expression and clinico-pathological characteristics of patients with CRC (n=181).

Features	n	Yes-associated protein −	+	χ^2 value	P-value
Age					
<60	79	20	59	0.104	0.747
≥60	102	28	74		
Sex					
Male	112	31	81	0.203	0.653
Female	69	17	52		
Differentiation					
High	44	20	24	10.740	**0.005**
Moderate	105	21	84		
Low	32	7	25		
T stage					
T1-T2	63	18	45	0.209	0.648
T3-T4	118	30	88		
Lymph node metastasis					
No	99	34	65	6.865	**0.009**
Yes	82	14	68		
Duke's stage					
A	26	15	11	23.901	**<0.0001**
B	53	19	34		
C+D	102	14	88		

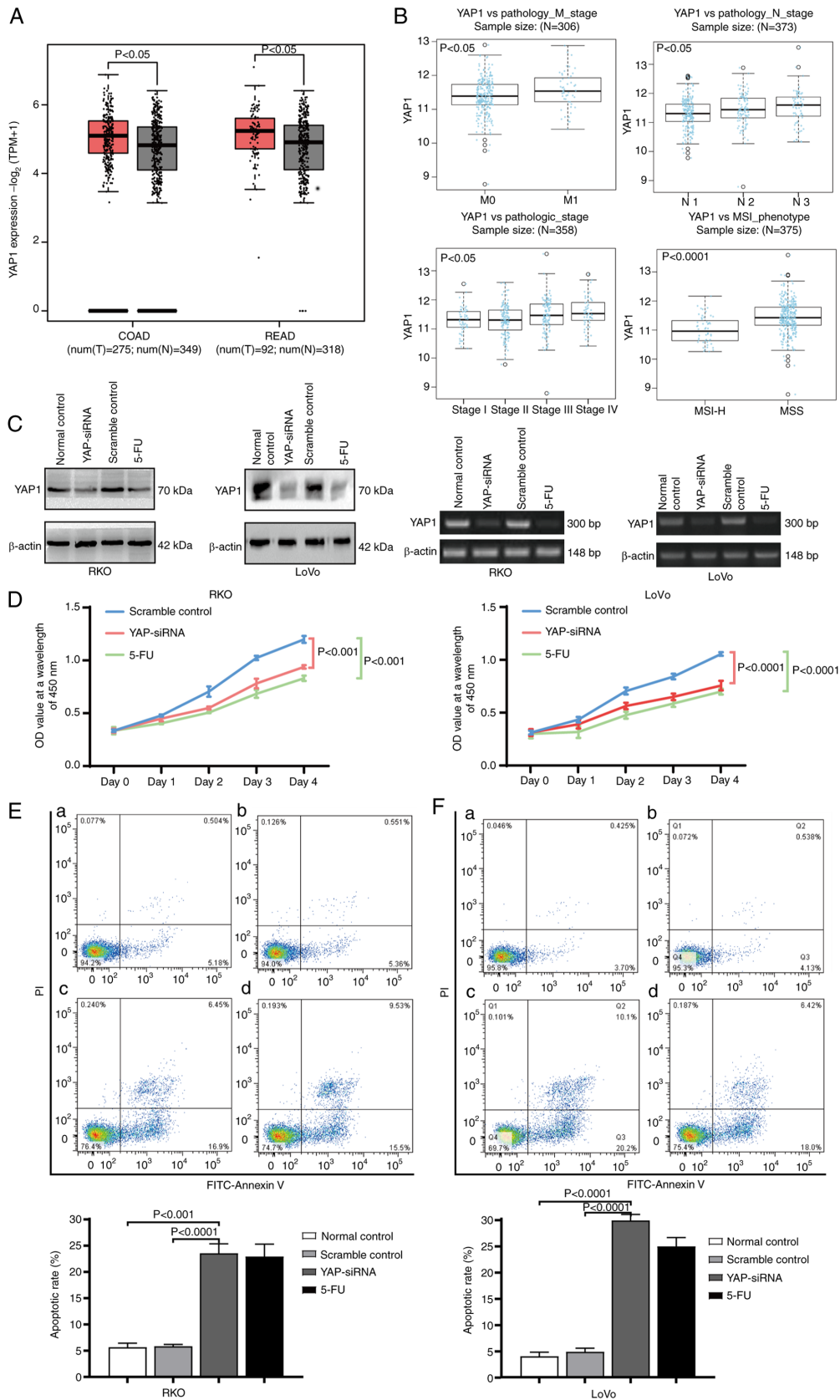Bold values indicate significant differences (P<0.05).

Figure 2. Expression of YAP protein is upregulated in CRC tissues, which is correlated with the aggressive characteristics of CRC. (A) Relative expression of YAP in CRC tissues and normal colorectal mucosa samples. (B) Correlation of YAP expression with M stage, pathological stage, microsatellite instability phenotype and N stage. (C) Validation of YAP expression in normal control group, YAP-siRNA group, scramble control group, and 5-FU group using western blot analysis and reverse transcription-quantitative PCR in RKO and LoVo cells. (D) RKO and LoVo cell proliferation detected by MTT assay after transfection with YAP-siRNA. (E) Rate of RKO cell apoptosis detected by flow cytometry 48 h after transfection with YAP-siRNA: (a) Normal control group, (b) Scramble control group, (c) YAP-siRNA transfected group, and (d) 5-FU group. (F) Rate of LoVo cell apoptosis detected by flow cytometry 48 h after transfection with YAP-siRNA: (a) Normal control group, (b) Scramble control group, (c) YAP-siRNA transfected group, and (d) 5-FU group. YAP, yes-associated protein; CRC, colorectal cancer; siRNA, small interfering RNA; 5-fluorouracil; MSS, microsatellite stable; MSI-H, microsatellite instability-high. YAP, yes-associated protein; CRC, colorectal cancer; siRNA, small interfering RNA; 5-fluorouracil; MSS, microsatellite stable; MSI-H, microsatellite instability-high.
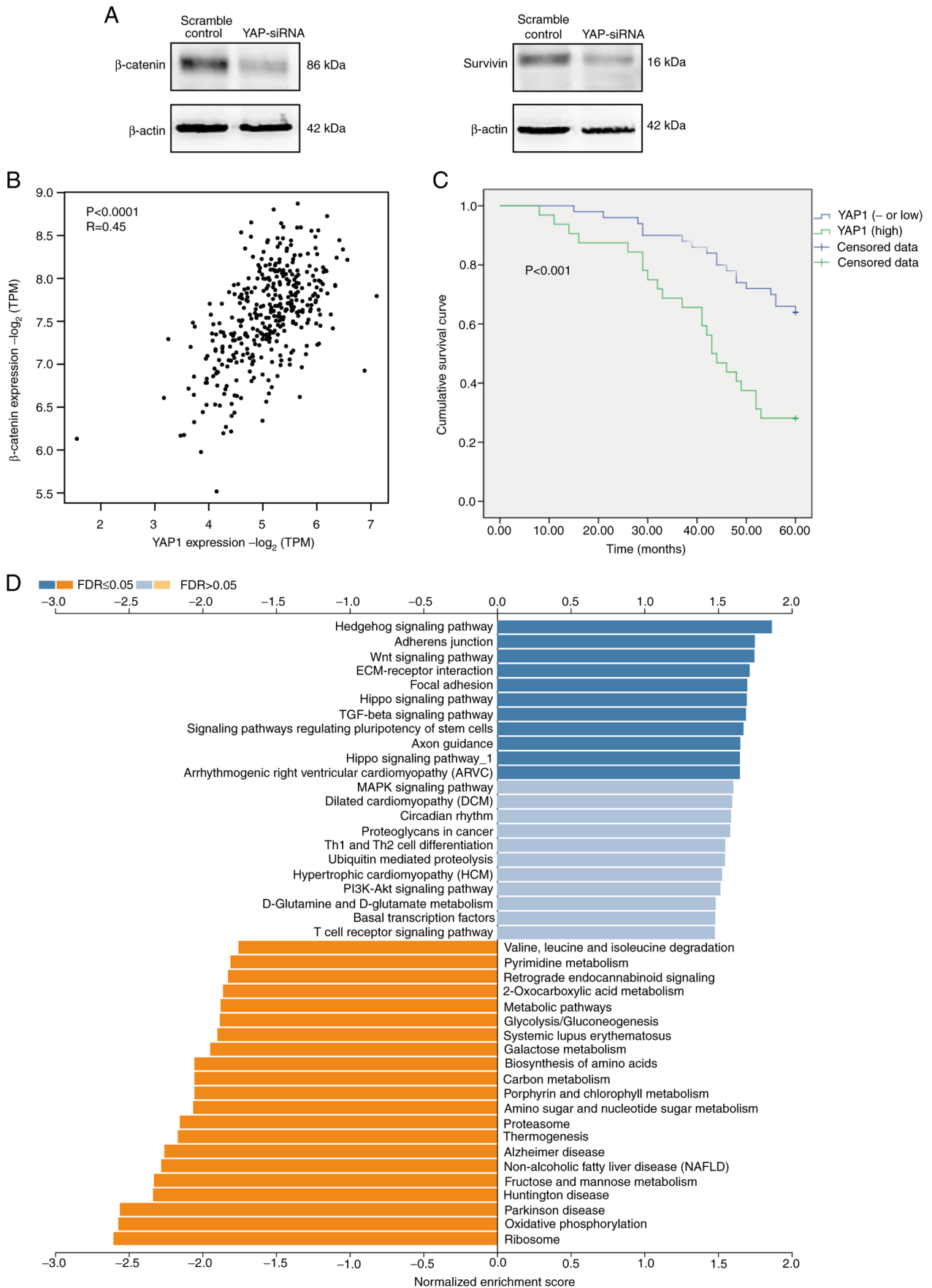
Figure 3. YAP is involved in the Hippo and Wnt/β-catenin signaling pathways in CRC. (A) β-catenin and baculoviral inhibitor of apoptosis repeat-containing 5/survivin in scramble control RKO and YAP-siRNA transfected RKO cells detected using western blot analysis. (B) Correlation between the relative expression of YAP and β-catenin. (C) Kaplan-Meier survival curves for patients with CRC according to YAP expression. (D) Kyoto Encyclopedia of Genes and Genomes pathway analysis based on Linkedomics showing that YAP expression is associated with the Hippo and Wnt/β-catenin signaling pathways in CRC. YAP, yes-associated protein; CRC, colorectal cancer; small interfering RNA; FDR, false discovery rate.

Table II. Relation between different location of YAP and clinicopathological parameters and β-catenin expression pattern in CRC.

Features	n=181	YAP - (48)	Low[a] (47/181)	High[b] (86/181)	χ^2 value	P-value	YAP - (48)	Cytoplasm (58/181)	Nucleus and nucleus/ plasma (75/181)	χ^2 value	P-value
Differentiation											
High	44	20	14	10	0.056	0.451	20	11	13	2.090	0.352
Moderate	105	21	25	59			21	34	50		
Low	32	7	8	17	16.816	**<0.0001**	7	13	12	4.422	0.110
Lymph node metastasis											
No	99	34	27	38	0.060	0.807	34	31	34	0.054	0.817
Yes	82	14	20	48	7.304	**0.007**	14	27	41	4.531	**0.003**
Duke's stage											
A	26	15	5	6	1.117	0.572	15	5	6	2.291	0.318
B	53	19	16	18			19	18	16		
C+D	102	14	26	62	17.332	**<0.0001**	14	35	53	11.030	**0.004**
β-catenin nucleus expression											
+	103	10	26	67	**0.019**	0.800	16	28	59	0.120	0.109
-	78	38	21	19	0.403	**<0.0001**	32	30	16	0.370	**<0.0001**
β-catenin cytoplasm expression											
+	45	8	7	30	0.137	0.067	11	13	21	**0.039**	0.603
-	136	40	40	56	0.221	**0.003**	37	45	54	0.061	0.414
β-catenin nucleus/ plasma expression											
+	22	10	5	7	**0.027**	0.713	7	9	6	0.066	0.378
-	159	38	42	79	0.117	0.117	40	50	69	0.107	0.152

Bold values indicate significant differences (P<0.05). [a]Cytoplasmic positive cells ≤50% and nuclear positive cells ≤ 10%. [b]Cytoplasmic positive cells > 50% and nuclear positive cells>10%. YAP, yes-associated protein.
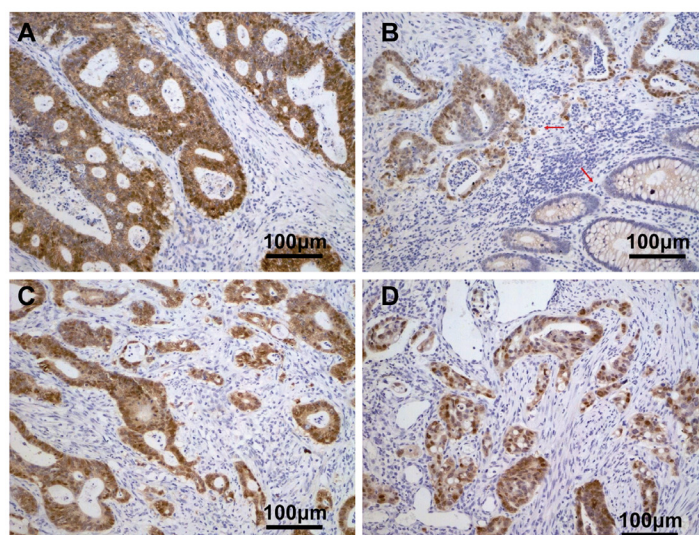

Figure 4. Expression profile of β-catenin protein in CRC tissues. (A) Cytoplasmic and nuclear staining of β-catenin was positive in well-differentiated CRC tissues. (B) Diffuse nuclear expression of β-catenin was positive in moderately differentiated CRC tissues (C) Cytoplasmic and nuclear staining of β-catenin was positive in moderate to poorly differentiated CRC tissues. (D) Nuclear expression of β-catenin was positive in poorly differentiated CRC tissues. CRC, colorectal cancer.

the normal control and scramble control groups, the apoptotic rates of YAP-siRNA RKO and LoVo group were increased by 11.35% (11.90±2.07%) and 16.9% (15.23±2.31%), respectively. The apoptotic rates in YAP-siRNA RKO and LoVo groups were significantly higher than those in the normal control and scramble control groups, and were similar to that of the 5-FU group (Fig. 2E).

YAP is involved in the Hippo and Wnt/β-catenin signaling pathways in CRC. Western blot analysis showed that compared with the scramble control group, β-catenin and BIRC5/survivin protein expression in RKO cells was reduced following transfection with YAP-siRNA (Fig. 3A). Bioinformatics analysis revealed that the expression levels of YAP and β-catenin were significantly correlated in CRC (Fig. 3B), in agreement with the western blot analysis. Immunohistochemical analysis showed that high YAP protein expression was significantly correlated with the expression of β-catenin in the nucleus and cytoplasm of CRC tissues, YAP expression in the nucleus and nuclear plasma was associated with the expression of β-catenin in the nucleus (P<0.05), (Fig. 4 and Table II). Conversely, there was no association between YAP expression in the nucleus and the expression of β-catenin in the cytoplasm or nuclear plasma (Fig. 4 and Table II). Also, Kaplan-Meier survival curves were used to characterize survival differences categorized by YAP expression, and patients with high YAP expression had a significantly lower mortality rate (n=32) compared with those with low YAP expression (n= 50) (P<0.001) (Fig. 3C). KEGG cluster analysis showed that differentially expressed genes with pathway annotation were mapped to six significant pathways (P<0.05; Fig. 3D), including the Hippo and Wnt/β-catenin signaling pathways. Consistent with the protein-protein interaction results, KEGG pathway analysis confirmed that these differentially expressed genes were primarily enriched in the Hippo and Wnt/β-catenin signaling pathways.

Discussion

In the present study, immunohistochemistry was used to detect the expression of YAP in 181 CRC tissues. The results indicated that the total positive expression rate of YAP was significantly higher in CRC tissues than in normal colorectal mucosa samples. Furthermore, the expression of YAP in CRC tissues was associated with histological differentiation, Duke's stage and lymph node metastasis, suggesting that aberrant YAP upregulation plays an important role in the occurrence and development of CRC. Previous studies have shown that patients with CRC with strong nuclear localization of YAP have a poor prognosis (25,26). The results of the present study showed that the expression of YAP in the nucleus and nuclear plasma was associated with Duke's stage. Although high levels of YAP expression were associated with histological differentiation, Duke's stage and lymph node metastasis, the cytoplasmic expression was not related to these clinical characteristics. This suggests that strong nuclear localization of YAP has greater clinical significance for the occurrence and progression of CRC.

The upregulation of YAP is reported to be an independent prognostic factor for some tumors, such as hepatocellular carcinoma (27). In the present study, the 5-year survival rate of patients in the high YAP expression group was significantly lower than that of patients in the low expression groups, suggesting that YAP plays an important role in the prognosis of CRC. Microsatellites are short tandem repeats that are distributed throughout the human genome. MSI refers to DNA methylation or gene mutations that cause mismatch repair gene deletions, resulting in the insertion or deletion of microsatellite repeats. The subsequent changes in length are closely related to the occurrence of tumors. Microsatellite stability is divided into three types: MSI-H, MSS and MSI-L. In terms of surgical efficacy, MSI-H is optimal, followed by MSS, with MSI-L being the worst. The current analysis showed that the expression level of YAP was higher in patients with MSS CRC than in those with MSI-H CRC. This lends further support for the use of YAP as an independent prognostic factor for the occurrence and progression of CRC.

In vitro studies were also conducted to determine the effect of YAP on the proliferation and apoptosis of RKO and LoVo cells. siRNA experiments showed that YAP increased proliferation and inhibited apoptosis in RKO and LoVo cells, suggesting a tumor promoting role of YAP in CRC. Furthermore, to the best of our knowledge, the present study is the first to show that the carcinogenesis of YAP may be due to the interaction between the Hippo and Wnt/β-catenin signaling pathways. The most important factor in the role of YAP in promoting the occurrence and progression of CRC may be the direct or indirect interactions of YAP with β-catenin. In the present study, YAP expression was found to be associated with the Wnt/β-catenin pathway, apoptosis and proliferation in colorectal cancer cell lines (RKO and LoVo). RKO is a poorly differentiated colon cancer cell line. RKO cells possess wild-type, but not mutant p53-type, and lack the human thyroid receptor nuclear receptor (28). The level of p53 protein in RKO cells was higher than that in RKO-E6 cells. The cell line formed tumors in nude mice and formed colonies in soft agar (29). Thus, experiments performed using the RKO cell line are relatively stable, and the experimental results are objective and reliable.

In conclusion, detecting the expression level of YAP and its nuclear expression pattern in CRC tissues is indicative of the prognosis of patients with CRC. The present study provides a theoretical basis for molecular targeted therapy in CRC, by targeting YAP.

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

LC and CZ designed the present study and wrote the paper. ZB, QW and JC conducted the experiments and carried out the statistical analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Research was conducted in accordance with the Declaration of Helsinki. The study design was approved by the Ethics Committee of Anhui Medical University. Written informed consent was obtained from all participants for the use of their clinical samples.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. DeSantis CE, Miller KD, Dale W, Mohile SG, Cohen HJ, Leach CR, Goding Sauer A, Jemal A and Siegel RL: Cancer statistics for adults aged 85 years and older, 2019. CA Cancer J Clin 69: 452-467, 2019.
2. Siegel RL, Miller KD, Goding Sauer A, Fedewa SA, Butterly LF, Anderson JC, Cercek A, Smith RA and Jemal A: Colorectal cancer statistics, 2020. CA Cancer J Clin 70: 145-164, 2020.
3. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA and Jemal A: Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 68: 394-424, 2018.
4. Garza Treviño EN, González PD, Valencia Salgado CI and Martinez Garza A: Effects of pericytes and colon cancer stem cells in the tumor microenvironment. Cancer Cell Int 19: 173, 2019.
5. Tang M, Wang H, Cao Y, Zeng Z, Shan X and Wang L: Nomogram for predicting occurrence and prognosis of liver metastasis in colorectal cancer: A population-based study. Int J Colorectal Dis 36: 271-282, 2021.
6. Zanconato F, Cordenonsi M and Piccolo S: YAP and TAZ: A signalling hub of the tumour microenvironment. Nat Rev Cancer 19: 454-464, 2019.
7. Yang R, Cai TT, Wu XJ, Liu YN, He J, Zhang XS, Ma G and Li J: Tumour YAP1 and PTEN expression correlates with tumour-associated myeloid suppressor cell expansion and reduced survival in colorectal cancer. Immunology 155: 263-272, 2018.
8. Pan Y, Tong JHM, Lung RWM, Kang W, Kwan JSH, Chak WP, Tin KY, Chung LY, Wu F, Ng SSM, et al: RASAL2 promotes tumor progression through LATS2/YAP1 axis of hippo signaling pathway in colorectal cancer. Mol Cancer 17: 102, 2018.
9. Moon S, Kim W, Kim S, Kim Y, Song Y, Bilousov O, Kim J, Lee T, Cha B, Kim M, et al: Phosphorylation by NLK inhibits YAP-14-3-3-interactions and induces its nuclear localization. EMBO Rep 18: 61-71, 2017.
10. Felley-Bosco E and Stahel R: Hippo/YAP pathway for targeted therapy. Transl Lung Cancer Res 3: 75-83, 2014.
11. Tsuji T, Ozasa H, Aoki W, Aburaya S, Yamamoto Funazo T, Furugaki K, Yoshimura Y, Yamazoe M, Ajimizu H, Yasuda Y, et al: YAP1 mediates survival of ALK-rearranged lung cancer cells treated with alectinib via pro-apoptotic protein regulation. Nat Commun 11: 74, 2020.
12. Yu S, Zhang Y, Li Q, Zhang Z, Zhao G and Xu J: CLDN6 promotes tumor progression through the YAP1-snail1 axis in gastric cancer. Cell Death Dis 10: 949, 2019.
13. Yan H, Li H, Li P, Li X, Lin J, Zhu L, Silva MA, Wang X, Wang P and Zhang Z: Long noncoding RNA MLK7-AS1 promotes ovarian cancer cells progression by modulating miR-375/YAP1 axis. J Exp Clin Cancer Res 37: 237, 2018.
14. Guo L, Chen Y, Luo J, Zheng J and Shao G: YAP1 overexpression is associated with poor prognosis of breast cancer patients and induces breast cancer cell growth by inhibiting PTEN. FEBS Open Bio 9: 437-445, 2019.
15. Yin MX and Zhang L: Hippo signaling in epithelial stem cells. Acta Biochim Biophys Sin (Shanghai) 47: 39-45, 2015.
16. Park J, Kim GH, Lee J, Phuong BTC, Kong B, Won JE, Won GW, Lee YH, Han HD and Lee Y: MST2 silencing induces apoptosis and inhibits tumor growth for estrogen receptor alpha-positive MCF-7 breast cancer. Toxicol Appl Pharmacol 408: 115257, 2020.
17. Hasegawa K, Fujii S, Matsumoto S, Tajiri Y, Kikuchi A and Kiyoshima T: YAP signaling induces PIEZO1 to promote oral squamous cell carcinoma cell proliferation. J Pathol 253: 80-93, 2021.
18. Liu Y, Wang X and Yang Y: Hepatic Hippo signaling inhibits development of hepatocellular carcinoma. Clin Mol Hepatol 26: 742-750, 2020.
19. Azar WJ, Christie EL, Mitchell C, Liu DS, Au-Yeung G and Bowtell DDL: Noncanonical IL6 signaling-mediated activation of YAP regulates cell migration and invasion in ovarian clear cell cancer. Cancer Res 80: 4960-4971, 2020.
20. Dey A, Varelas X and Guan KL: Targeting the Hippo pathway in cancer, fibrosis, wound healing and regenerative medicine. Nat Rev Drug Discov 19: 480-494, 2020.
21. Ma K, Xu Q, Wang S, Zhang W, Liu M, Liang S, Zhu H and Xu N: Nuclear accumulation of Yes-associated protein (YAP) maintains the survival of doxorubicin-induced senescent cells by promoting survivin expression. Cancer Lett 375: 84-91, 2016.
22. Kriz V and Korinek V: Wnt, RSPO and Hippo signalling in the intestine and intestinal stem cells. Genes (Basel) 9: 20, 2018.
23. Kahn M: Can we safely target the WNT pathway? Nat Rev Drug Discov 13: 513-532, 2014.
24. 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.
25. Camargo FD, Gokhale S, Johnnidis JB, Fu D, Bell GW, Jaenisch R and Brummelkamp TR: YAP1 increases organ size and expands undifferentiated progenitor cells. Curr Biol 17: 2054-2060, 2007.
26. Zhou D, Zhang Y, Wu H, Barry E, Yin Y, Lawrence E, Dawson D, Willis JE, Markowitz SD, Camargo FD and Avruch J: Mst1 and Mst2 protein kinases restrain intestinal stem cell proliferation and colonic tumorigenesis by inhibition of Yes-associated protein (Yap) overabundance. Proc Natl Acad Sci USA 108: E1312-E1320, 2011.
27. Li H, Wang S, Wang G, Zhang Z, Wu X, Zhang T, Fu B and Chen G: Yes-associated protein expression is a predictive marker for recurrence of hepatocellular carcinoma after liver transplantation. Dig Surg 31: 468-478, 2014.
28. van Bree C, Franken NA, Snel FA, Haveman J and Bakker PJ: Wild-type p53-function is not required for hyperthermia-enhanced cytotoxicity of cisplatin. Int J Hyperthermia 17: 337-346, 2001.
29. Nagashima M, Shiseki M, Pedeux RM, Okamura S, Kitahama-Shiseki M, Miura K, Yokota J and Harris CC: A novel PHD-finger motif protein, p47ING3, modulates p53-mediated transcription, cell cycle control, and apoptosis. Oncogene 22: 343-350, 2003.