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Original Article

Expression of Yes-associated protein in liver cancer and its correlation with clinicopathological features and prognosis of liver cancer patients

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Abstract: Objective: To investigate the expression of Yes-associated protein (YAP) in liver cancer and its correlation with clinicopathological features and prognosis of liver cancer patients. Methods: A total of 95 patients receiving surgery due to liver cancer were recruited. Results: In 95 liver cancers, YAP expression was significantly higher than that in adjacent normal tissues. In addition, of liver cancers, 14.7% was negative for YAP (14/95), 29.5% (28/95) weakly positive, 21.1% (20/95) positive and 34.7% (33/95) strong positive, and low expression and high expression were observed in 44.2% (42/95) and 55.8% (53/95) of liver cancers, respectively. Of adjacent normal tissues, 13.7% (13/95) were negative or weakly positive for YAP. The mean survival time of patients with high YAP expression was significantly longer than that of patients with low YAP expression (Log-rank = 9.206, $P < 0.01$). Univariate analysis showed portal vein thrombosis ($P < 0.01$), metastasis ($P < 0.01$), American Joint Committee on Cancer Staging (AJCC) stage ($P < 0.01$), alpha fetoprotein (AFP) ($P < 0.01$) and high YAP expression ($P < 0.01$) were factors affecting the overall survival of liver cancer patients. However, multivariate analysis showed metastasis ($P < 0.01$) and high YAP expression ($P < 0.01$) were independent risk factors of overall survival of liver cancer patients. Conclusion: YAP expression increases significantly in liver cancer and it may be involved in the occurrence and development of liver cancer. YAP expression is an independent risk factor affecting the overall survival of liver cancer patients.

Keywords: Liver cancer, YAP, survival analysis, prognosis

Introduction

Primary liver cancer remains the fifth most common malignancy and the third leading cause of cancer-related death. About 0.5 million people is diagnosed with liver cancer annually worldwide [1, 2]. The occurrence and development of liver cancer are complicated pathophysiological processes related to multiple factors with involvement of multiple steps. The causes of liver cancer identified to date include hepatitis B, hepatitis C, alcoholic cirrhosis and smoking. The occurrence of liver cancer is occult, and it is highly malignant and presents rapid progression. Usually, liver cancer is at the advanced stage in a majority of patients at the time of diagnosis when surgical intervention is infeasible and traditional chemotherapy and/or radiotherapy has a poor efficacy [3]. Thus, patients with advanced liver cancer have an extremely poor prognosis and the interval from the time of diagnosis to death is no longer than 6 months [4]. Thus, early identification, early diagnosis and early therapy are important to cure liver cancer and increase the survival rate. To identify molecular target used in the early diagnosis of liver cancer is of great importance.

Yes-associated protein (YAP) is a major downstream effector of classic Hippo pathway and may be phosphorylated by Wnt resulting in its inactivation [5]. Hippo pathway is an anti-tumor pathway identified in drosophila in recent years. Later, it has been confirmed that Hippo pathway is also highly conservative and a classic anti-tumor pathway [6, 7]. YAP, a downstream molecule of Hippo pathway, is a classic oncogene. To

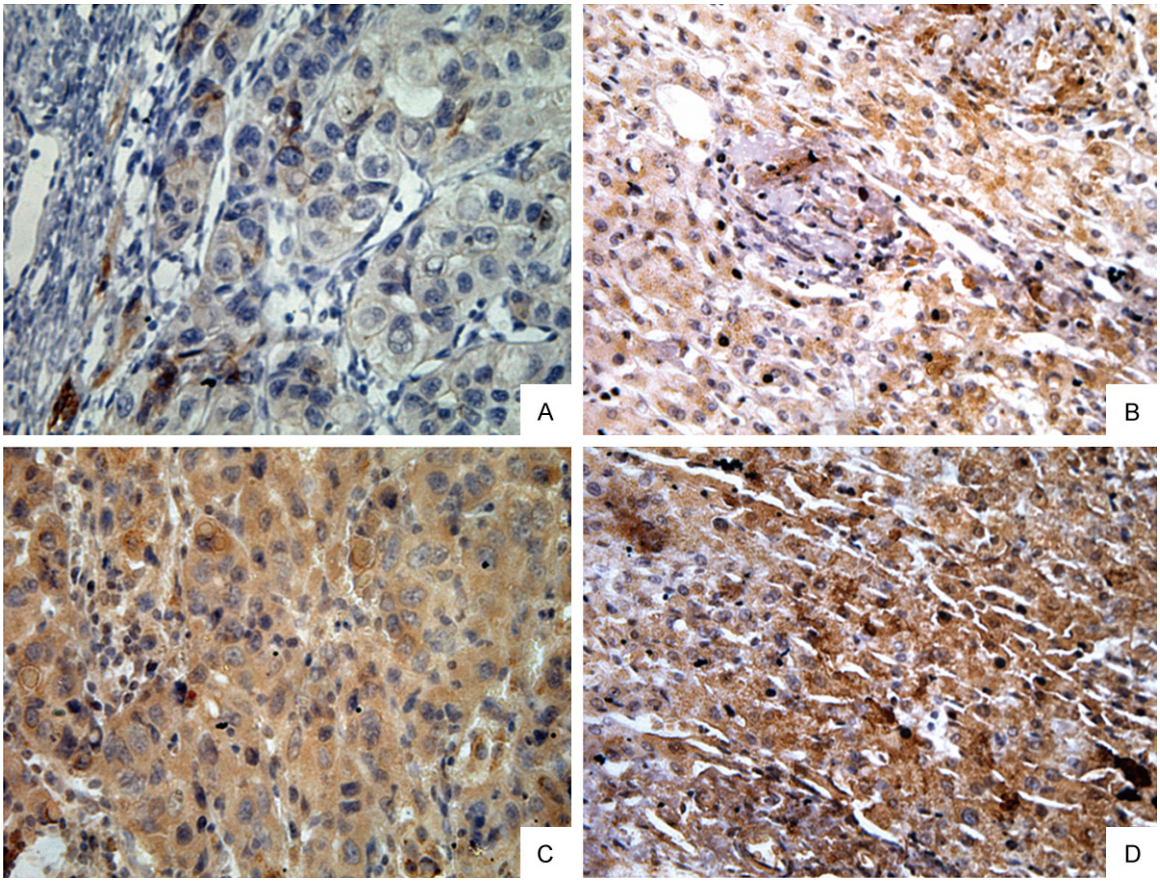

Figure 1. YAP expression in liver cancer. A. Negative; B. Weakly positive; C. Positive; D. Strongly positive (400 ×).

date, several studies have confirmed that YAP expression increases significantly in breast cancer, endometrial cancer, non-small cell lung cancer, ovarian cancer, stomach cancer, liver cancer, and so on [8-13]. To date, several studies have also revealed that YAP activation is closely associated with the occurrence and development of liver cancer. Perra et al found YAP expression increased at early stage of liver cancer in humans and the increased YAP expression was closely related to the proliferation of liver cancer cells [14]. In addition, there is evidence showing that YAP may interact with other proteins to promote the development of liver cancer [15-17]. However, whether YAP is related to the prognosis of liver cancer patients after surgery is still poorly understood.

In this study, 95 liver patients receiving surgery were recruited, and the liver cancer tissues and adjacent normal tissues were harvested for immunohistochemistry for YAP, aiming to explore the correlation of YAP expression in liver cancer with clinicopathological features and prognosis of liver cancer patients.

Materials and methods

Patient characteristics

A total of 95 patients receiving surgery due to liver cancer were recruited from July 2008 to July 2012. There were 66 males and 29 females. The median age was 65 years (range: 38-76 years). Chemotherapy and radiotherapy were not performed before surgery, and postoperative pathological examination confirmed the diagnosis of liver cancer. This study was approved by the Ethics Committee of our hospital and conducted in accordance with the principles of Helsinki Declaration. Informed consent was obtained before study. The clinicopathological features including age, gender, tumor diameter, number of tumors, degree of differentiation, satellite foci, portal venous thrombosis, metastasis, American Joint Com-

Table 1. Correlation of YAP expression in liver cancer with clinicopathological features (n = 95)

Clinicopathological features	n	YAP expression		χ^2	*P* value
		High (%) (Score 5-12)	Low (%) (Score 0-4)		
Total number	95				
Gender					
M	66	30 (45.5)	36 (54.5)	0.318	0.573
F	29	15 (51.7)	14 (48.3)		
Age (yr)					
≤ 60	49	23 (46.9)	26 (53.1)	0.016	0.9
> 60	46	21 (45.7)	25 (54.3)		
Tumor diameter (cm)					
≤ 5	50	9 (18)	41 (82)	41.83	< 0.001
> 5	45	38 (84.4)	7 (15.6)		
Number of tumors					
One	60	29 (48.3)	31 (51.7)	0.687	0.407
More than one	35	20 (57.1)	15 (42.9)		
Degree of differentiation					
Poorly to moderately	74	54 (73)	20 (27)	6.642	0.01
Well	21	9 (42.9)	12 (57.1)		
Satellite foci					
No	70	34 (48.6)	36 (51.4)	0.963	0.326
Yes	25	15 (60)	10 (40)		
Portal vein thrombosis					
No	73	33 (45.2)	40 (54.8)	0.156	0.693
Yes	22	11 (50)	11 (50)		
Metastasis					
No	69	20 (29)	49 (71)	17.802	< 0.001
Yes	26	20 (76.9)	6 (23.1)		
AJCC stage					
I-II	36	10 (27.8)	26 (72.2)	21.569	< 0.001
III-IV	59	45 (76.3)	14 (23.7)		
AFP (μg/L)					
≤ 400	29	13 (44.8)	16 (55.2)	7.689	< 0.001
> 400	66	49 (74.2)	17 (25.8)		

Footnotes: AJCC, American Joint Committee on Cancer.

mittee on Cancer Staging (AJCC), alpha fetoprotein (AFP) were recorded.

Reagents

YAP rabbit anti-human polyclonal antibody (#4912; Cell Signaling Technology, USA; 1:250) was diluted with 5% non-fat milk. Sodium citrate solution was purchased from Beyoutime Biotech. SABC immunohistochemistry kit and DAB kit were purchased from Vector Laboratories (USA).

Immunohistochemistry with SABC method

After surgery, the liver cancer tissues were embedded in paraffin and sectioned into 5-μm sections. 1) Sections were deparaffinized in xylene, followed by hydration and washing in PBS thrice (5 min for each). 2) Antigen retrieval: The citrate sodium solution was diluted with double-distilled water into 1 × citrate sodium solution and sections were boiled in this solution for 2 h, followed by washing in PBS thrice (5 min for each). 3) Sections were blocked in 3% hydrogen peroxide for 15 min to inactivate peroxidase, followed by washing in phosphate buffered saline (PBS) thrice (5 min for each). 4) Sections were blocked at room temperature for 1 h with 1% goat serum in 5% BSA and 0.1% Tween 20, and excess solution was removed; 5) Sections were treated with primary antibody at 4°C overnight; 6) On the second day, sections were allowed to stay at room temperature for 45 min, and the primary antibody solution was removed by washing in PBS thrice (5 min for each). 7) Sections were treated with biotinylated secondary antibody at room temperature for 1 h and the secondary antibody was removed by washing in PBS thrice (5 min for each), 8) SABC solution was prepared at 0.5 h before use and sections were treated with SABC solution for 0.5 h. The solution was then removed by washing in PBS thrice (5 min for each); 9) Visualization was done with 3,3'-diaminobenzidine (DAB) and then sections were washed with water to stop the reaction; (10) After washing in distilled water, sections were counterstained with hematoxylin for 2 min and then treated with alcohol in hydrochloric acid. 10) Sections were dehydrated with ethanol and transparentized

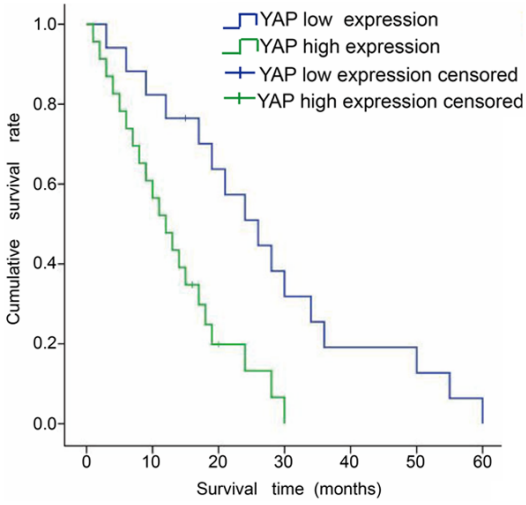

Figure 2. Survival analysis of liver cancer patients with low or high YAP expression.

with xylene and mounted with neutral gum. Then, sections were observed under a microscope. In negative control group, the primary antibody was replaced with PBS, and the positive control antibody was provided by the manufacturer.

Determinations

Positive results were observed in positive control group and negative results in negative control group suggest reliable staining. Cells with brown or yellow-brown granules were regarded positive for YAP, and detection of YAP expression was done by two pathologists independently. The YAP positive area and the staining intensity were semi-quantified as follows and averages were obtained: 1) YAP positive area: proportion of positive cells of < 5%, 6%-25%, 26%-50%, 50%-75% and > 75% was defined as scores 0, 1, 2, 3, and 4, respectively; 2) Staining intensity: no staining, light yellow staining, brown staining and yellow-brown staining were defined as scores 0, 1, 2 and 3, respectively. The product of positive area and staining intensity was classified as 0, negative; 1-4, weakly positive; 5-8, positive; 9-12, strong positive. Low expression was defined as scores 0-4 and high expression as scores 5-12.

Follow up

Patients were followed up by telephone or hospital visit, and the mean period of follow up was 15 months. Follow up was initiated immediately after surgery and terminated at the end of July 2013. For dead patients, the follow up was terminated at the time of death. Of these patients, 3 were lost to follow up, and the time to loss to follow up was defined as the period of follow up.

Statistical analysis

Statistical analysis was performed with SPSS version 17.0. Pearson chi square test was employed to analyze the correlation of YAP expression with clinicopathological features. Kaplan-Meier method was used to delineate the survival curve, and survival analysis was performed with log-rank test. COX regression model was employed for univariate and multivariate analyses. A value of $P < 0.05$ was considered statistically significant.

Results

YAP expression in liver cancer

YAP was mainly expressed in the nucleus and a little of YAP in the cytoplasm. Thus, positive staining was mainly confined to the nucleus. Of 95 liver cancers, 14.7% (14/95) was negative for YAP, 29.5% (28/95) weakly positive, 21.1% (20/95) positive and 34.7% (33/95) strongly positive. In addition, low YAP expression was observed in 44.2% (42/95) of liver cancers and high YAP expression in 55.8% (53/95) (**Figure 1**). The adjacent normal tissues were negative or weakly positive for YAP.

Correlation of YAP expression with clinicopathological features of liver cancer patients

Of 95 patients, the YAP expression was positively related to tumor diameter ($\chi^2 = 41.83$, $P < 0.01$), degree of tumor differentiation ($\chi^2 = 6.642$, $P < 0.05$), metastasis ($\chi^2 = 17.802$, $P < 0.01$), AJCC stage ($\chi^2 = 21.569$, $P < 0.05$) and AFP ($\chi^2 = 7.689$, $P < 0.05$), but not with gender ($\chi^2 = 0.318$, $P = 0.573$), age ($\chi^2 = 0.016$, $P = 0.9$), number of tumors ($\chi^2 = 0.687$, $P = 0.407$), satellite ($\chi^2 = 0.963$, $P = 0.326$) and portal vein thrombosis ($\chi^2 = 0.156$, $P = 0.693$) (**Table 1**).

Survival analysis

The survival time of 95 patients was subjected to analysis with Kaplan-Meier method. Results showed the 2-year survival rate was 13%, and mean survival time was 13.3 months (median: 12 months) in patients with high YAP expres-

Table 2. Univariate analysis of factors affecting overall survival of liver cancer patients (n = 95)

Variable	B	SE	RR	95% CI	*P* value
Gender	0.015	0.235	1.015	0.641-1.607	0.95
Age	0.355	0.225	1.426	0.918-2.214	0.113
Tumor diameter	0.245	0.214	1.277	0.84-1.942	0.252
Number of tumors	0.262	0.219	1.299	0.846-1.955	0.231
Degree of differentiation	0.29	0.251	1.337	0.817-2.186	0.248
Satellite foci	1.313	0.237	1.14	0.717-1.814	0.58
Portal vein thrombosis	1.005	0.263	2.731	1.632-4.573	< 0.01
Metastasis	1.379	0.256	3.969	2.403-6.557	< 0.01
AJCC stage	1.128	0.239	3.09	1.933-4.94	< 0.01
AFP	0.482	0.238	1.619	1.016-2.581	< 0.05
YAP positive	1.101	0.151	3.009	2.237-4.047	< 0.01

Footnotes: AJCC, American Joint Committee on Cancer.

Table 3. Multivariate analysis of factors affecting overall survival of liver cancer patients (n = 95)

Variable	B	SE	RR	95% CI	*P* value
Gender	-0.46	0.28	0.631	0.364-1.094	0.101
Age	0.161	0.246	1.175	0.726-1.903	0.512
Tumor diameter	0.258	0.263	1.295	0.773-2.168	0.326
Number of tumors	0.331	0.276	1.393	0.811-2.393	0.23
Degree of differentiation	0.138	0.309	1.148	0.626-2.106	0.655
Satellite foci	0.076	0.265	1.1079	0.642-1.813	0.774
Portal vein thrombosis	0.1	0.319	1.105	0.592-2.065	0.754
Metastasis	0.969	0.364	2.636	1.291-5.382	< 0.01
AJCC stage	0.579	0.353	1.784	0.894-3.561	0.101
AFP	0.391	0.267	1.479	0.877-2.496	0.143
YAP positive	0.798	0.232	2.222	1.410-3.5	< 0.01

Footnotes: AJCC, American Joint Committee on Cancer.

sion. In patients with low YAP expression, the 2-year survival rate was 51% and the mean survival time was 27.3 months (median: 26 months). The mean survival time of patients with high YAP expression was significantly shorter than that of patients with low YAP expression (Log-rank = 9.206, P < 0.01; **Figure 2**).

COX regression analysis

Univariate analysis and multivariate analysis were performed for all the clinicopathological features. Univariate analysis showed factors affecting the overall survival included portal vein thrombosis (P < 0.01), metastasis (P < 0.01), AJCC stage (P < 0.01), AFP (P < 0.05) and high YAP expression (P < 0.01) (**Table 2**). However, multivariate analysis revealed metastasis (P < 0.01) and high YAP expression (P < 0.01) were independent factors affecting the overall survival of liver cancer patients (**Table 3**).

Discussion

To date, some factors (such as TNM stages, grades, degree of differentiation, metastasis, concomitant hepatitis, cirrhosis and surgical factors [surgical efficacy, blood transfusion, blood loss, time of portal clamping]) have been used to predict the prognosis of liver cancer patients, and subsequent therapeutic measures are determined according to these predictors [18]. However, patients with same pathological stage, grade or degree of differentiation present different prognosis. This suggests that liver cancer with same pathological stage, grade and degree of differentiation has different biological behaviors, and predicting the prognosis on the basis of above factors still has limitations. Tumor markers such as AFP heterogeneity (AFP-L3), Phosphatidylinositol proteoglycan-3 (GPC-3), Golgi protein-73 (GP-73), heat shock protein (HSP70), osteopontin (OPN) and human zinc finger protein (hZNF23) have been used in the early diagnosis of liver cancer. Nevertheless, they still have limitations in the evaluation of prognosis of liver cancer patients [19]. Thus, to identify biological markers which can be used to reflect the malignant biological behaviors and guide the prediction of the prognosis of liver cancer patients is imperative.

YAP is a downstream effector molecule of Hippo signaling pathway and also a transcriptional coactivator. It can promote the transcription of multiple genes and has been a classic oncogene [20]. The expression of YAP and P-YAP is dependent on the molecular phenotype of cancers. In breast cancer, YAP is highly expressed in the cytoplasm and nucleus and can be used

to determine the prognosis of breast cancer patients [8]. YAP may serve as a transcriptional factor to regulate the proliferation and apoptosis. In endometrial cancer, YAP was found to promote cell proliferation, and YAP expression in the nucleus could serve as a prognostic factor and increase the sensitivity to radiotherapy.

Some investigators speculate that YAP may not only promote the proliferation and invasion of liver cancer cells, but interact with other proteins (especially the oncogenes CREB, MEK1, C-MYC and Trib2) to facilitate the occurrence and development of liver cancer [15-17]. Liu et al postulated that YAP was highly expressed in transitional cell carcinoma of the bladder and closely related to the proliferation and invasion of cancer cells and could serve as a molecular biological marker to predict the prognosis of transitional cell carcinoma of the bladder [21]. The role of increased YAP expression in liver cancer in the prediction of prognosis of liver cancer patients after surgery is still unclear.

This study aimed to investigate the YAP expression and its correlation with clinicopathological features of liver cancer patients. Immunohistochemistry showed YAP expression in liver cancer increased significantly when compared with adjacent normal tissues. In liver cancer patients, YAP expression was related to the portal vein thrombosis, metastasis, AJCC stage and AFP, which are important factors related to the clinical stages and grades. These indicate that YAP is involved in the occurrence and development of liver cancer and the prognosis of liver cancer patients. Kaplan-Meier survival analysis showed the 2-year survival rate was 51% in patients with low YAP expression and 13% in patients with high YAP expression, suggesting that the higher the YAP expression, the poorer the prognosis. Univariate analysis and multivariate analysis showed YAP expression was associated with the prognosis of liver cancer patients and served as an independent predictor of overall survival. High YAP expression is a risk factor of liver cancer patients. Above findings suggest that YAP may serve as a predictor of metastasis and recurrence of liver cancer and can be used to guide the individualized therapy. The specific mechanism and the interaction between YAP and other oncogenes are required to be investigated in future studies.

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Disclosure of conflict of interest

None.

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References

[1] Fernandez-Rodriguez CM and Gutierrez-Garcia ML. Prevention of hepatocellular carcinoma in patients with chronic hepatitis B. World J Gastrointest Pharmacol Ther 2014; 5: 175-182.

[2] Liu C, Duan LG, Lu WS, Yan LN, Xiao GQ, Jiang L, Yang J and Yang JY. Prognosis evaluation in patients with hepatocellular carcinoma after hepatectomy: comparison of BCLC, TNM and Hangzhou criteria staging systems. PLoS One 2014; 9: e103228.

[3] Tu ZQ, Li RJ, Mei JZ and Li XH. Down-regulation of long non-coding RNA GAS5 is associated with the prognosis of hepatocellular carcinoma. Int J Clin Exp Pathol 2014; 7: 4303-4309.

[4] Zhang BH, Yue XQ and Huang WH. Factors predicting survival in patients with advanced hepatocellular carcinoma. J Four Mili Med Univ 2005; 26: 661-664.

[5] Xu F, Zhang J and Ma D. [Crosstalk of Hippo/YAP and Wnt/beta-catenin pathways]. Yi Chuan 2014; 36: 95-102.

[6] Zhao B, Li L, Wang L, Wang CY, Yu J and Guan KL. Cell detachment activates the Hippo pathway via cytoskeleton reorganization to induce anoikis. Genes Dev 2012; 26: 54-68.

[7] Zhao B, Li L, Lu Q, Wang LH, Liu CY, Lei Q and Guan KL. Angiomotin is a novel Hippo pathway component that inhibits YAP oncoprotein. Genes Dev 2011; 25: 51-63.

[8] Kim SK, Jung WH and Koo JS. Yes-associated protein (YAP) is differentially expressed in tumor and stroma according to the molecular subtype of breast cancer. Int J Clin Exp Pathol 2014; 7: 3224-3234.

[9] Tsujiura M, Mazack V, Sudol M, Kaspar HG, Nash J, Carey DJ and Gogoi R. Yes-associated protein (YAP) modulates oncogenic features and radiation sensitivity in endometrial cancer. PLoS One 2014; 9: e100974.

[10] Lin XY, Zhang XP, Wu JH, Qiu XS and Wang EH. Expression of LATS1 contributes to good prog-

nosis and can negatively regulate YAP oncoprotein in non-small-cell lung cancer. Tumour Biol 2014; 35: 6435-6443.
[11] Xia Y, Chang T, Wang Y, Liu Y, Li W, Li M and Fan HY. YAP promotes ovarian cancer cell tumorigenesis and is indicative of a poor prognosis for ovarian cancer patients. PLoS One 2014; 9: e91770.
[12] Jiao S, Wang H, Shi Z, Dong A, Zhang W, Song X, He F, Wang Y, Zhang Z, Wang W, Wang X, Guo T, Li P, Zhao Y, Ji H, Zhang L and Zhou Z. A peptide mimicking VGLL4 function acts as a YAP antagonist therapy against gastric cancer. Cancer Cell 2014; 25: 166-180.
[13] Wang J, Wang H, Zhang Y, Zhen N, Zhang L, Qiao Y, Weng W, Liu X, Ma L, Xiao W, Yu W, Chu Q, Pan Q and Sun F. Mutual inhibition between YAP and SRSF1 maintains long non-coding RNA, Malat1-induced tumourigenesis in liver cancer. Cell Signal 2014; 26: 1048-1059.
[14] Perra A, Kowalik MA, Ghiso E, Ledda-Columbano GM, Di Tommaso L, Angioni MM, Raschioni C, Testore E, Roncalli M, Giordano S and Columbano A. YAP activation is an early event and a potential therapeutic target in liver cancer development. J Hepatol 2014; 61: 1088-96.
[15] Xiao W, Wang J, Ou C, Zhang Y, Ma L, Weng W, Pan Q and Sun F. Mutual interaction between YAP and c-Myc is critical for carcinogenesis in liver cancer. Biochem Biophys Res Commun 2013; 439: 167-172.
[16] Li L, Wang J, Zhang Y, Zhang Y, Ma L, Weng W, Qiao Y, Xiao W, Wang H, Yu W, Pan Q, He Y and Sun F. MEK1 promotes YAP and their interaction is critical for tumorigenesis in liver cancer. FEBS Lett 2013; 587: 3921-3927.
[17] Wang J, Ma L, Weng W, Qiao Y, Zhang Y, He J, Wang H, Xiao W, Li L, Chu Q, Pan Q, Yu Y and Sun F. Mutual interaction between YAP and CREB promotes tumorigenesis in liver cancer. Hepatology 2013; 58: 1011-1020.
[18] Fang JY and Dong Y. Clinical factors affecting prognosis of liver cancer patients. Guid Chin Med 2013; 363-364.
[19] Xiao L, Yang Y, Mao R and Bao YX. New molecular markers of cancers used for diagnosis of liver cancer and prediction of prognosis. Onco Prog 2012; 10: 141-144.
[20] Liu AM, Xu Z and Luk JM. An update on targeting Hippo-YAP signaling in liver cancer. Expert Opin Ther Targets 2012; 16: 243-247.
[21] Liu JY, Li YH, Lin HX, Liao YJ, Mai SJ, Liu ZW, Zhang ZL, Jiang LJ, Zhang JX, Kung HF, Zeng YX, Zhou FJ and Xie D. Overexpression of YAP 1 contributes to progressive features and poor prognosis of human urothelial carcinoma of the bladder. BMC Cancer 2013; 13: 349.