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Overexpression of SMYD3 Is Predictive of Unfavorable Prognosis in Hepatocellular Carcinoma

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SET and MYND domain-containing protein 3 (SMYD3) is a kind of histone lysine methyltransferase, responsible for transcriptional activation as a member of an RNA polymerase complex. The ectopic expression of SMYD3 is proved to promote the progress of many kinds of cancers. In hepatocellular carcinoma (HCC), SMYD3 was demonstrated to promote the proliferation and metastasis of HCC cell lines, but the clinical significance of SMYD3 has not been elucidated. In the present study, we detected the expression of SMYD3 in 100 HCC tissues with immunohistochemistry and divided these tissue specimens into high-expression group and low-expression group according to the immunohistochemical score of SMYD3. Importantly, the intensity of SMYD3 immunoreactivity was significantly stronger in HCC tissues than that in adjacent normal tissues. Moreover, high expression levels of SMYD3 were significantly associated with larger tumor size (P = 0.043), suggesting that SMYD3 could promote the proliferation of HCC. Moreover, patients with positive hepatitis B virus infection had higher expression levels of SMYD3 (P = 0.013). With univariate and multivariate analysis, we explored the prognostic significance of SMYD3 in HCC. As a result, high expression levels of SMYD3 were significantly correlated to the poorer clinical outcome of HCC patients (P = 0.009) and were identified as an independent risk factor of HCC for predicting the unfavorable prognosis. In conclusion, overexpression of SMYD3 is an independent prognostic risk of unfavorable prognosis of HCC. We propose that the anti-SMYD3 therapy may be a potential approach to treat HCC.

Keywords: biomarker; hepatocellular carcinoma; prognosis; SET and MYND domain-containing protein 3; survival rate

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Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer and causes the third cancer-related death worldwide (Torre et al. 2015). There are about 782,500 new HCC cases and 745,500 HCC-related deaths worldwide in 2012, which is more severe in China (Xiao et al. 2016). The curative treatments of HCC include surgical resection, tumor ablation and liver transplantation. Thanks to the improvement of these treatments and other progresses made in adjacent therapies such as transcatheter arterial chemoembolization, the survival rate of patients with HCC shows an impressive increase (Bruix and Sherman 2011). However, the outcome for patients with HCC remains unsatisfactory. One reason to the unfavorable survival of HCC is its poor response to chemotherapy or radiotherapy. The poor response to adjuvant therapy partially resulted in the high recurrence. The identified targeted drug of HCC is Sorafenib, which only benefits approximate one third patients. More targeted drugs of HCC are still in urgent need and the discovery of these drugs is based on the exploration of effective biomarkers.

As a histone lysine methyltransferase, SET and MYND domain-containing protein 3 (SMYD3) belongs to SET and MYND-domain family (SMYD family) and plays an important role in transcriptional activation as a member of an RNA polymerase complex. The SMYD family consists of five members characterized by a structural feature that the catalytically active SET domain is split into an N-terminal portion via the intervention of the MYND domain. SMYD3 could di- and tri-methylate the lysine-4 of histone H3 and lysine-5 and lysine-20 of histone H4, thus regulating gene transcription through the histone methyltransferase activity (Van Aller et al. 2012). SMYD3 is

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widely expressed and its normal function is essential for fundamental biological process like proliferation. However, its ectopic expression was also reported to be associated with cancer progression. Many cancer-promoting genes could be potentiated by SMYD3 (Sarris et al. 2016), and the up-regulation of SMYD3 is observed and correlated with poor prognosis in many kinds of cancers, including breast cancer, lung adenocarcinoma, and pancreatic ductal adenocarcinomas (Giakountis et al. 2017).

In previous studies, SMYD3 was proved to promote HCC cell proliferation and invasion with experiments *in vitro and vivo* (Sarris et al. 2016). Intriguingly, the expression of SMYD3 was mainly in the cytoplasm in lung and pancreatic cancer cells (Mazur et al. 2014). Previous reports explored the expression and location of SMYD3 in HCC tissues but did not get a consensus. SMYD3 expression was reported to exist mainly in cytoplasm in HCC tissue with immunohistochemistry (He et al. 2012), but subcellular localization of SMYD3 was altered by the density of cultured Huh7 HCC cells (Hamamoto et al. 2004). SMYD3 was suggested to localize mainly in the cytoplasm at G0/G1 phase, and in the nuclei at S phase and G2/M phase (Hamamoto et al. 2004). Till now, the clinical and prognostic significance of SMYD3 in HCC tissues has not been well elucidated. In our study, we detected the expression of SMYD3 in HCC tissues with immunohistochemistry (IHC) and further evaluated the prognostic value of SMYD3 with univariate and multivariate analysis.

Materials and Methods

Patients and cohorts

The basic cohort in our study consisted of 232 patients diagnosed as HCC and underwent radical resection of HCC from 2006 to 2014 in Yidu Central Hospital. The testing cohort was selected out from the basic cohort following the criteria: (1) no pre-operational or post-operational adjuvant therapy, (2) available specimens for IHC, and (3) available follow-ups and medical records. The validation cohort was consisted of 100 cases, comprising of 9 female and 91 male patients with the average age as 49 years old. The average follow-up time of testing cohort was 34.5 months, ranging from 4.0-81.0 months. The clinical stage of HCC was identified referring to the 7th AJCC/UICC TNM classification system. The histopathological grade of tumor differentiation was evaluated referring to the Edmondson grading system. The overall survival time was identified as the time from operation to the death or the last follow-up time. Our study was approved by the Ethics Committee of Yidu Central Hospital, and the specimens were obtained with prior consent of patients' family with signed informed consent.

Immunohistochemistry

The expression of SMYD3 was detected with the formalin-fixed, paraffin-embedded specimens according to previous studies (Xu et al. 2014). Briefly, samples were de-waxed in xylene and rehydrated in graded alcohol first. Then the slides were incubated in sodium citrate solution (pH 6.0) boiled with microwave for optimal antigen retrieval and in 3% H_2O_2 for 15 minutes to block the endogenous peroxidase activity. After incubation in 5% bovine serum albumin for 30 minutes, primary antibody of SMYD3 (Abcam, Cambridge, UK) was used to incubate the specimens at 4°C overnight, and the corresponding biotinylated secondary antibody was applied to incubate the tissues at room temperature for 2 hours. Antigen visualization was achieved with DAB (3, 3- diaminobenzidine) for 15 min. The slides were finally counterstained with 1% hematoxylin.

Immunohistochemical evaluation

The results of IHC were evaluated by two independent pathologists who were blinded to the clinicopathological information. The results of immunohistochemistry were semi-quantified with the final score, which was the arithmetic product of staining intensity multiplied by the positive cell percentage according to previous studies (Xu et al. 2017; Liu et al. 2017a). The staining intensity was ranked as follows: score 0 represented negative staining, score 1 for weak staining, score 2 for median staining and score 3 for strong staining; and percentage of positive-stained tumor cells was scored as score 1 for < 25% of positive cells; score 2 for 25%-50% of positive cells; score 3 for 50%-75% of positive cells; and score 4 for 75%-100% of positive cells. Thus, the final IHC score, which was staining intensity multiplied by positive cell percentage, ranged from 0 to 12. The cut-off of IHC score divided the cohort into low SMYD3 expression group and high SMYD3 expression group. The cut-off was determined by the receiver operating characteristic (ROC) curve and defined as the point with the highest sum of the specificity plus sensitivity according to previous report (Liu et al. 2017b). In our study, the calculated cut-off was 5.4, which means cases with IHC score ≥ 6 was set as high SMYD3 expression group.

Statistical analysis

All data in our study were analyzed using SPSS 22.0 software (Chicago, IL, USA) without special instruction. The correlations between the expression of SMYD3 and other clinicopathological parameters were analyzed with Chi-Square test. The survival curves were represented with the Kaplan-Meier method. The differences between the groups were calculated with log-rank test. The independent prognostic factor was identified with the Cox regression hazard model. $P < 0.05$ was defined as statistically significant in our study.

Results

Expression of SMYD3 in HCC tissues

The location of SMYD3 varies depending on the HCC specimens. However, in our study, the SMYD3 immunoreactivity was mainly observed in nuclei of HCC tumor cells. The representative IHC images of high expression of SMYD3 and low expression of SMYD3 are displayed in Fig. 1. Moreover, SMYD3 expression in 20 pairs of HCC tissues and their corresponding adjacent tissues were compared. The SMYD3 staining intensity was significantly stronger in HCC tissues than that in normal tissues (Fig. 2). There were 56 patients with low expression levels of SMYD3 and 44 patients with high expression levels of SMYD3, accounting for 56.0% and 44.0 % of the total cohort, respectively.

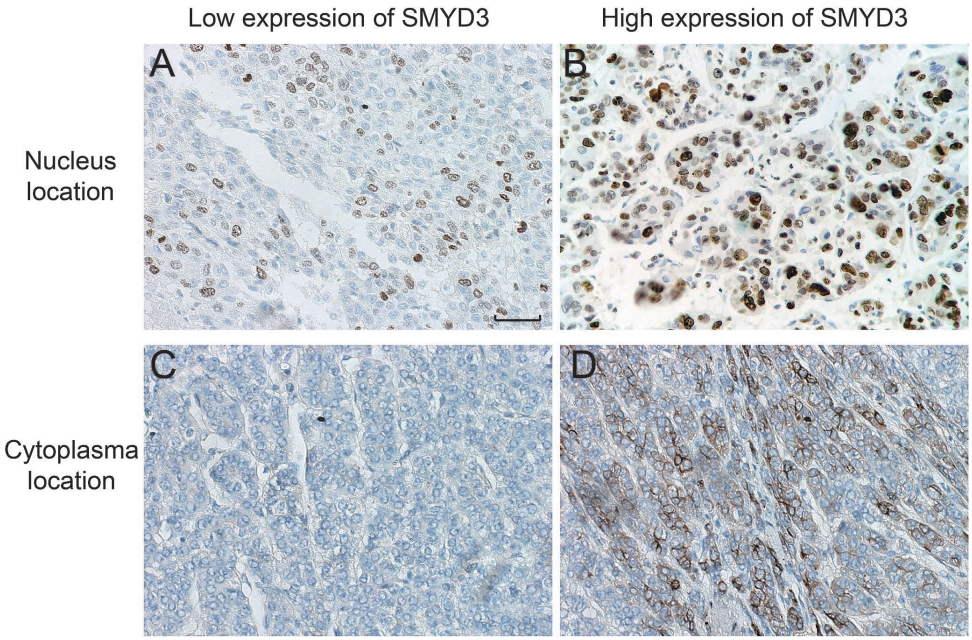

Fig. 1. Representative immunohistochemical figures.
(A) The representative image for low-expression levels of SMYD3 with nucleus localization. The total IHC score was 4 and defined as low expression. The histological grade was highly differentiated. Scale bar: 50 μm. (B) The representative image for high-expression levels of SMYD3 with nucleus localization. The total IHC score was 12 and defined as high expression. The histological grade was highly differentiated. (C) The representative image for low-expression levels of SMYD3 with cytoplasmic localization. The total IHC score was 0 and defined as low expression. The histological grade was highly differentiated. (D) The representative image for high-expression levels of SMYD3 with cytoplasmic localization. The total IHC score was 9 and defined as high expression. The histological grade was highly differentiated.

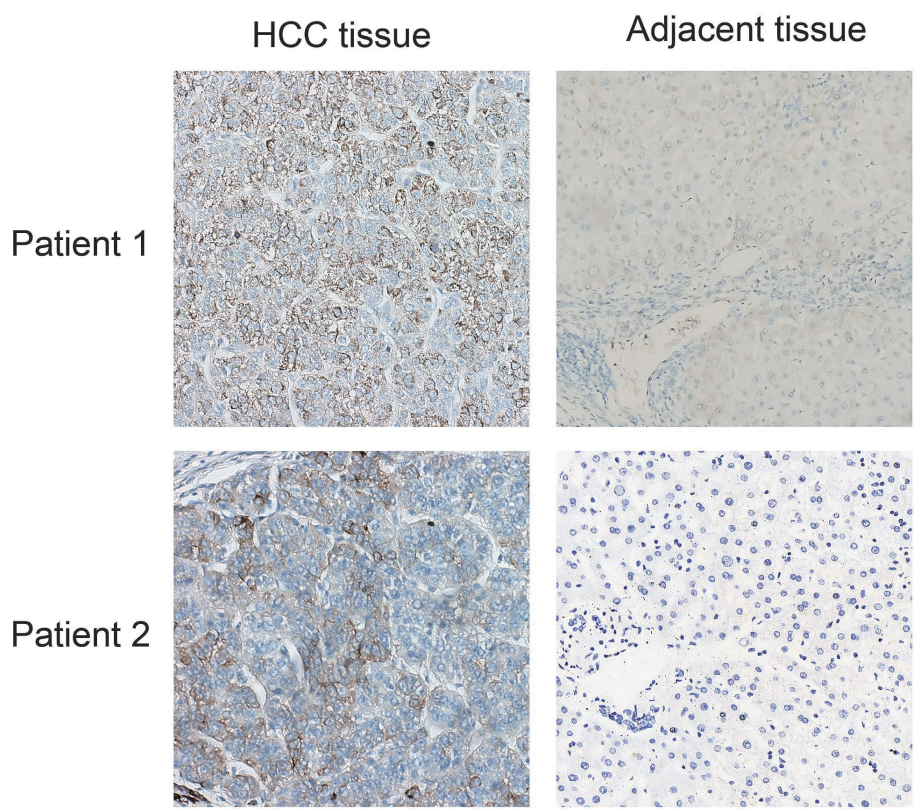

Fig. 2. The SMYD3 expression in the HCC tissues and the corresponding adjacent tissues.
(A) and (B) Representative IHC images of the HCC tissue and adjacent tissue (Patient 1). (C) and (D) Representative IHC images of the HCC tissue and adjacent tissue (Patient 2).

Association between SMYD3 and clinicopathological factors

Chi-square test was used to calculate the association between SMYD3 expression and clinicopathological factors including patients' sex, age, tumor size, tumor number, histopathological grade, hepatitis B virus (HBV) and hepatitis C virus (HCV) infection (Table 1). In our analysis, the SMYD3 expression was significantly related to the tumor size ($P = 0.043$). Patients with high expression of SMYD3 had higher probability to present with larger tumor size, indicating that SMYD3 could promote the HCC cell proliferation. Previous studies have demonstrated that SMYD3 could interact with the proteins of HBV and HCV (Eberle et al. 2014; Hayashi et al. 2016). We thus investigated the correlation between SMYD3 expression and infection of HBV/HCV. Interestingly, higher SMYD3 expression levels were observed in patients with HBV infection (P = 0.013). By contrast, such correlation was not observed in patients

Table 1. Correlations between clinicopathological factors and SMYD3 expression.

Characters	number	percentage	SMYD3		P*
			Low	High	
Sex					
Female	9	9.0%	5	4	0.978
Male	91	91.0%	51	40	
Age					
< 50	38	38.0%	23	15	0.537
≥ 50	62	62.0%	33	29	
Tumor size (cm)					
≤ 5	41	41.0%	28	13	0.043
> 5	59	59.0%	28	31	
Tumor number					
Single	91	91.0%	49	42	0.292
Multiple	9	9.0%	7	2	
Histopathological grade					
I	8	8.0%	3	5	0.542
II	53	53.0%	26	27	
III	29	29.0%	17	12	
HBsAg					
Negative	31	31.0%	23	8	0.013
Positive	69	69.0%	33	36	
HCV					
Negative	98	98.0%	51	41	0.698
Positive	8	69.0%	5	3	
T stage					
I	13	13.0%	9	4	0.528
II	38	38.0%	23	15	
III	45	45.0%	22	23	
IV	4	4.0%	2	2	
N stage					
N0	98	98.0%	54	44	0.502
N1	2	2.0%	2	0	
TNM stage					
I	13	13.0%	9	4	0.219
II	38	38.0%	23	15	
III	47	47.0%	22	25	
IV	2	2.0%	2	0	

*means calculated by χ^2 test.
SMYD3, SET and MYND domain-containing protein 3.

with HCV, which could be partially attributed to the small sample size of patients with HCV. In China, most patients suffered with HCC because of the infection of HBV instead of HCV, which was different from Western Countries. There were no other clinicopathological factors proved to be significantly associated with SMYD3 expression in our cohort.

Correlation between SMYD3 expression and survival rate

The prognostic value of SMYD3 in HCC was evaluated with univariate analysis and multivariate analysis. Univariate analysis with Kaplan-Meier method was applied to evaluate the correlation between the clinicopathological factors including the SMYD3 expression and the 5-year overall survival rate (Table 2). The statistical significance was generated with the log-rank test. The expression of SMYD3 was remarkably correlated to the clinical outcome of HCC patients (P = 0.009). The 5-year overall survival rates of high expression of SMYD3 and low expression of SMYD3 were 29.6% and 48.7%, respectively (Fig. 3A). Moreover, larger tumor size (> 5 cm), advanced T stage (III + IV) and advanced TNM stage (III + IV) were all significantly related to poorer prognosis of HCC (P = 0.006, P < 0.001 and P < 0.001, respectively (Fig. 3B-D). The statistical significance of T stage and TNM stage was similar, because very few patients with lymphatic invasion or

Table 2. Correlation between SMYD3 and survival rates.

Characters	5-year survival rate(%)	P
Sex		
Female	66.7	0.127
Male	37.2	
Age		
< 50	39.2	0.588
≥ 50	44.4	
Tumor size (cm)		
≤ 5	54.2	0.006
> 5	30.5	
Tumor number		
Single	39.2	0.997
Multiple	44.4	
Histopathological grade		
I+II	40.9	0.758
III	37.9	
HBsAg		
Negative	36.0	0.588
Positive	51.8	
HCV		
Negative	40.1	0.695
Positive	37.5	
T stage		
I+II	59.5	< 0.001
III+IV	20.2	
N stage		
N0	40.7	0.383
N1	0.0	
TNM stage		
I+II	59.5	< 0.001
III+IV	20.2	
SMYD3		
Low	48.7	0.009
High	29.6	

*means calculated by log-rank test.
SMYD3, SET and MYND domain-containing protein 3.

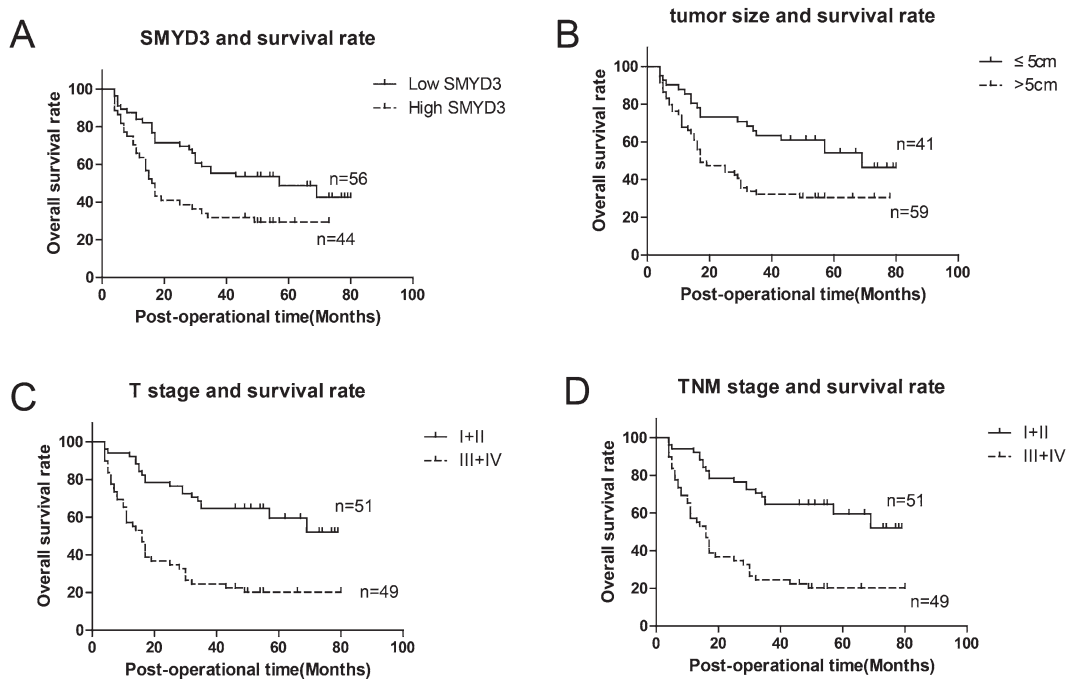

Fig. 3. Overall survival curves of SMYD3, tumor size, T stage and TNM stage.
The overall survival curves of pateints were displayed with Kaplan-Meier method and stratified with (A) SMYD3 expression, (B) tumor size, (C) T stage and (D) TNM stage. The statistical difference was calculated by log-rank test.

metastasis received radical resection of HCC and enrolled into our cohort; namely, patients with advanced T stage were highly overlapped with patients in advanced TNM stage. The survival curves of T stage and TNM stage were also similar.

The multivariate analysis with Cox-regression hazard model was applied to identify the independent prognostic factors. All the prognostic factors which have been identified by univariate analysis were enrolled into the Cox-regression hazard model (Table 3). TNM stage was excluded because of the obvious interaction with T stage. The high expression of SMYD3 ($P = 0.029$, HR = 1.81) and advanced T stage ($P < 0.001$, HR = 2.3) were demonstrated as independent prognostic factors of HCC, which could predict unfavorable prognosis of HCC patients.

Table 3. Multivariate analysis.

Characters	HR	95% CI	P
Tumor size (cm)			
≤ 5	1		
> 5	0.9	0.47-1.74	0.756
T stage			
I+II	1		
III+IV	2.3	1.51-3.67	< 0.001
SMYD3			
Low	1		
High	1.8	1.06-3.07	0.029

*means calculated by Cox-regression model.
SMYD3, SET and MYND domain-containing protein 3; HR, hazard ratio; 95% CI, 95% confidence interval.

Discussion

Histone modification regulates the chromatin structure and transcriptional activation and repression. The defects of histone lysine methyltransferases also play a prominent role in the control of critical cellular processes, such as proliferation, apoptosis, cell differentiation, inflammation and DNA repair (Fullgrabe et al. 2010; Rugg-Gunn et al. 2010; Black et al. 2012; Alabert et al. 2015). There are important and solid reports elucidating SMYD3's function in liver and colon cancer cells. Overexpression of SMYD3 can promote HCC cell proliferation and invasion via regulating the transcription of some oncogenes (Hamamoto et al. 2004; Sarris et al. 2016). Hamamoto et al. (2004) showed that SMYD3 up-regulation caused cell proliferation in cell lines. Moreover, SMYD3 was demonstrated to promote transcription of several downstream proliferaion-involved gene such as *Wnt10B* and *PIK3CB* via binding to the promoter region and the H3-methyltransferase activity. However, there were no previous studies focused on the clinical significance of SMYD3 in HCC; namely, whether SMYD3 expression can lead to advanced stage or poor prognosis is unknown. In our study, we show that high expression of SMYD3 is associated with larger tumor size, which is consistent in part with in vitro experiments showing that SMYD3 could accelerate cell proliferation (Hamamoto et al. 2004).

Effective prognostic biomarkers are helpful for the exploration of therapeutic strategies and many biomarkers for the prediction and intervention of HCC have been inves-

tigated. The demonstration of the oncogenic role of vascular endothelial growth factor receptor (VEGFR) in HCC leads to the development of Sorafenib, prolonging the life span of HCC patients. Emerging proofs indicated the oncogenic function of SMYD3 in HCC. SMYD3 is demonstrated to be a transcriptional potentiator of multiple cancer-promoting genes and promote the HCC progression like cell proliferation and epithelial-mesenchymal transition (Sarris et al. 2016). The histone H3 lysine 4 has been proved to be a key substrate of SMYD3 in SMYD3-induced HCC progression, and the upregulation of trimethylated histone H3 lysine 4 is associated with poor prognosis of HCC (Hamamoto et al. 2004; He et al. 2012). These lines of evidence suggested SMYD3 as a potential drug target in HCC. Fortunately, a small-molecule inhibitor of SMYD3 is available and exhibits the ability to impair cancer cell growth (Peserico et al. 2015). Our findings, SMYD3 as a prognostic biomarker of HCC, can facilitate the studies on exploring SMYD3-targeted drugs for HCC treatment and may contribute to expanding the patients' survival time.

In our study, we observed that SMYD3 is mainly detected in the nucleus, but the subcellular location of SMYD3 was not exclusively nuclear. In HCC, the fluctuation of SMYD3 intracellular localization between the cytoplasm and the nucleus was influenced by the cell cycle phase (Hamamoto et al. 2004; Giakountis et al. 2017). Nuclear translocation of SMYD3 is usually during S phase and G2/M phase and can be considered as a reflection of proliferation (Hamamoto et al. 2004). The molecular mechanism that regulates the intracellular localization of SMYD3 is not well known, but it is generally accepted that SMYD3 regulates different cancer-related genes expression by different mechanisms in different cancer types.

In summary, we investigated the expression of SMYD3 in HCC tissues with IHC and further evaluated its clinical significance. As the results, we showed that high expression levels of SMYD3 were correlated to larger tumor size (> 5 cm). Moreover, the upregulation of SMYD3 expression was significantly associated with unfavorable prognosis of HCC. High expression levels of SMYD3 can be identified as an independent prognostic factor predicting poorer prognosis of HCC. These results suggest that SMYD3 is a potential drug target of HCC, and further studies on SMYD3 may help explore a new treatment therapy.

Conflict of Interest

The authors declare no conflict of interest.

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