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Original Article

Expression of HDAC4 in hepatocellular carcinoma and its correlation with prognosis

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Abstract: The aim of this study was to elaborate the expression of HDAC4 in hepatocellular carcinoma and correlation with prognosis as well as clinical parameters. We chose a hepatocellular carcinoma tissue microarray with follow-up information (containing 90 hepatocellular carcinoma specimens). Immunohistochemistry was applied to investigate the expression level of HDAC4. The relationship between HDAC4 expression and the prognosis of early or later stage hepatocellular carcinoma were statistically analyzed by SPSS software respectively. HDAC4, expressed specifically in the cytoplasm, and also obviously higher in hepatocellular carcinoma tissues than para-carcinoma tissues (P<0.05). In the group of early hepatocellular carcinoma (stage1/stage2), the expression of HDAC4 was not correlated with ki67 expression (P=0.157); HDAC4 overexpression had better prognosis, although the *p* value was not significant (81.8% VS 48.3%, P=0.057). The expression of ki67 was not correlated with prognosis in this group (P=0.933). In the group of advanced hepatocellular carcinoma (stage3/stage4), the expression of HDAC4 was significantly correlated with ki67 expression (r=0.434, P=0.005); these two proteins were significantly negative correlation with prognosis in this group respectively (0% VS 27.3%, P=0.003; 0% VS 28.6%, P=0.000), COX multifactors analysis indicated that ki67 and M stage were independent predictors of this group (p=0.046, p=0.028). Conclusions: We hypothesized that HDAC4 might be regulated by different genes and perform different functions in different stages of hepatocellular carcinoma. In the early stage hepatocellular carcinoma patients, HDAC4 might participate an unknown tumor suppress network and prolong the survival time of patients; in the later stage of hepatocellular carcinoma, HDAC4 might be involved in the ki67 signaling network and indirectly promote the proliferation of hepatocellular carcinoma cells and reduced the survival time of patients. Because of the complexity of the biological function of HDAC4 in hepatocellular carcinoma, it might not all HCC patients can benefit from HDAC4 inhibitors in the treatment, it is necessary to do a further study on the clinical application of HDAC4 inhibitors.

Keywords: HDAC4, Ki67, hepatocellular carcinoma, prognosis, targeted therapy, immunohistochemistry

Introduction

Hepatocellular carcinoma (HCC) is the most common primary malignant tumor of the liver. In all tumors, the incidence of hepatocellular carcinoma is fifth, and mortality is fourth [1, 2]. China has become one of the world's high incidences of liver cancer because of 10% proportion of chronic hepatitis B [3]. HCC patients have a poor prognosis, the prognosis of HCC is worse than most cancers even after surgery or chemotherapy. Therefore, if can find new molecular markers for HCC prognosis and precise treatment, take different treatment modalities for different types of liver cancer patients, might improve the clinical therapeutic effect and reduce mortality.

The deacetylation of histone is one of the most important regulation of gene expression, histone deacelyase (HDAC) participated in this process. HDAC can bind with negatively charged DNA through histone acetylation function, and inhibit gene transcription. Expression of many proteins which related to cancer can be regulated by HDAC, it plays an important role in cancer development and considered to be a class of cancer drug target [4]. There are 18 members in human HDAC family, divided into four kinds [5]. HDAC4 is a member of class II HDAC, was upregulated in most tumors and played a role in promoting cancer. For example, AJ Wilson used siRNA to silence HDAC4 expression in colorectal cancer cells can inhibit cell growth and proliferation, and induce apoptosis

[6]. Wang HG found that using sodium butyrate can inhibit expression of HDAC4 protein in hepatoma cells, also can inhibit the hepatoma cell proliferation, migration and invasion significantly [7]. These results showed that HDAC4 is an oncogene through promote the proliferation of tumor cells. However, there are few reports about the prognostic relevance of HDAC4 and cancer especially on the prognosis of hepatocellular carcinoma. Therefore, the experiment carefully chose a hepatocellular carcinoma tissue microarray which contained 90 cases with follow-up information, IHC experiments was applied to study the correlation of HDAC4 expression and prognosis of HCC; meanwhile, this experiment added ki67 clinical immunohistochemistry data because previous studies have shown that inhibition of HDAC4 expression can inhibit cancer cell proliferation and ki67 is currently used the most extensive tumor cell proliferation marker, to detailed analysis of HDAC4 and ki67 expression, included the correlation between them and HCC occurrence, development, prognosis.

Materials and methods

Hepatocellular carcinoma tissue microarray

Hepatocellular carcinoma tissue microarray (HLiv-HCC180Sur-04) was obtained from Shanghai Outdo Biotech Co., Ltd, contained 90 carcinoma tissues and paired para-carcinoma tissues. Tissue microarray production, all donor paraffin-embedded sections were resected and stained by hematoxylin-eosin (HE). Then, the pathologist labelled typical pathological sites on HE slices. Using tissue microarray instrument (Beecher Instruments. Inc.) drilled 180 blocks on the blank recipient paraffin (diameter was 1.5 mm), and then set the target tissue core according to the position of HE. Subsequently, slicer (Leica, Germany) took continuous slices from tissue section to thickness of 4 um. Slices were attached to anti-off microslides.

The follow-up of HCC patients: The operation time was from January 2007 to November 2009 and the eventual follow-up time in September 2013, which followed 3.8-6.7 years. During this follow-up time, 57 patients were died of HCC, with a median follow-up time of 14 months (1-69 months); 33 patients were still alive, with a median follow-up time of 57 months (49-80 months). All patients were clinicopathologically diagnosed as hepatocellular carcinoma and received no extra treatment before surgery.

Immunohistochemistry

Two-step immunohistochemistry assay by DAKO Auto Stainer LinK48 was used: After antigen retrieval using citrate buffer, the tissue sections were blocked with goat serum and subsequently incubated with primary antibody which anti-HDAC4 (1:700, sc-46672, Santa Cruz) at 4°C overnight. Then, the tissue sections were incubated with secondary antibody (HRP-labeled anti-mouse antibody, DAKO). Washed with PBS, visualizing using diaminobenzidine (DAB) system and hematoxylin redying, observed and analyzed with microscope, randomly 3 high-magnification fields were chosen under optical microscope and calculated more than 3 × 100 cells. Scored and grouped with staining intensity, “Negative” is 0, “0-1+” for 0.5 points, “1+” for 1, “2+” for 2, “2+-3+” for 2.5, “3+” for 3. Samples less than 1.5 were divided into low expression group, and >1.5 were divided into high expression group. Statistical analysis was performed by SPSS software (version 17.0).

Statistical analyses

We divided the patients into two groups according to the clinical stage, there were 40 cases of stage1 and stage2, named early HCC group; and 42 cases of stage3 and stage4, named advanced HCC group; another 8 cases missed clinical stage information, were not included in the statistical analysis. Associations between HDAC4 expression in carcinoma tissues and para-carcinoma tissues were analyzed by paired NPar test. The correlation between HDAC4 expression and clinicopathological parameters of HCC was calculated by Spearman’s correlation analysis. Univariate analysis between HDAC4, Ki67 and survival time was evaluated using the Kaplan-Meier method and the log-rank test. Then, statistically significant variables in Univariate analysis would be included in COX multivariate regression survival analysis. $P<0.05$ was considered to be statistically significant.

Results

The expression pattern of HDAC4 in HCC and adjacent tissues

The results of Immunohistochemistry indicated that HDAC4 was localized in the cytoplasm in

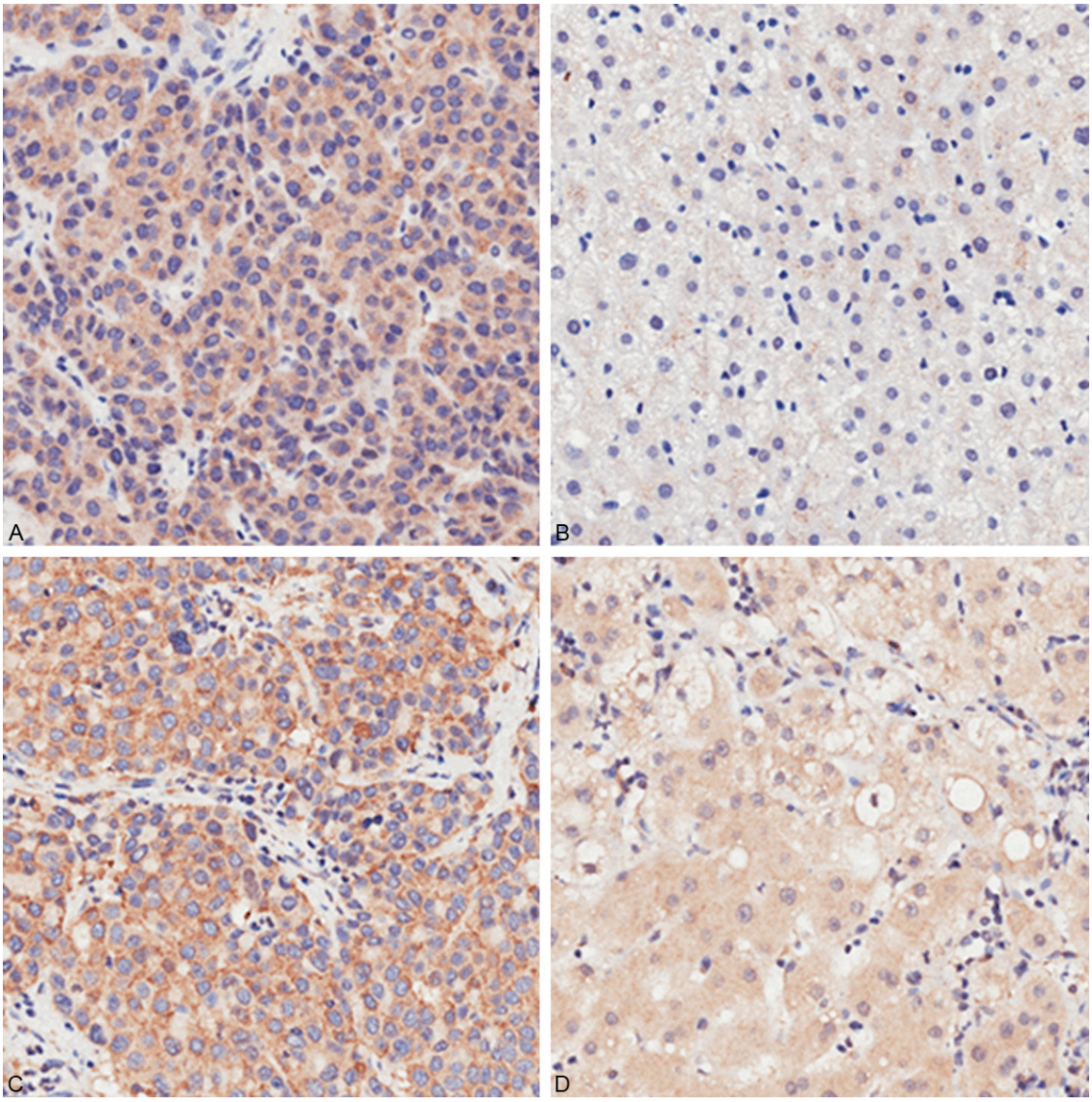

Figure 1. Immunohistochemistry of HDAC4: HDAC4 was specifically expressed in the cytoplasm in all HCC specimens. HDAC4 was higher in HCC tissues than that in para-carcinoma tissues in both early HCC group (A for HCC tissue, B for para-carcinoma tissue) and advanced HCC group (C for HCC tissue, D for para-carcinoma tissue) (Magnification times: ×200).

Table 1. The expression pattern of HDAC4 in HCC tissues and para-carcinoma tissues

HDAC4 expression intensity	HCC tissue	para-carcinoma tissue	*P* value
Early HCC (stage 1 and 2)	1.244±0.547	0.744±0.274	0.000
Advanced HCC (stage 3 and 4)	1.202±0.654	0.797±0.249	0.001

all HCC specimens. The representative pictures of the immunohistochemistry were shown in **Figure 1**. The data analyzed by paired NPar test revealed that positive staining rate of HDAC4 was higher in HCC tissues than that in para-carcinoma tissues in both early HCC group (P=0.000) and advanced HCC group (P=0.001). The analysis was showed in **Table 1**.

The correlation between HDAC4 expression and clinical index

The information which included in the statistical analysis of HCC patients were as follows: 40 cases of patients divided into early HCC group (stage1/stage2). Among them, 35 males, 5 females; age distribution from 26 to 73 years

Table 2. Correlation analysis of clinical data and HDAC4 expression in HCC

Clinical Data	Early HCC (40 cases)	HDAC4 low expression (29 cases)	HDAC4 high expression (11 cases)	Correlation coefficient	*P* value	Advanced HCC (42 cases)	HDAC4 low expression (33 cases)	HDAC4 High expression (9 cases)	Correlation coefficient	*P* value
Gender				0.056	0.731				-0.254	0.105
Male	35	25	10			39	30	9		
Female	5	4	1			3	3	0		
Ages				0.121	0.458				0.007	0.964
≤60	30	22	8			31	24	7		
>60	10	7	3			10	8	2		
Lost	0	0	0			1	1	0		
Tumor size				0.086	0.597				0.000	1.000
≤6 cm	31	23	8			5	4	1		
>6 cm	9	6	3			36	28	8		
Lost	0	0	0			1	1	0		
Pathological grading				-0.181	0.265				0.228	0.146
Grade I	3	3	0			0	0	0		
Grade II	27	17	10			23	19	4		
Class III	10	9	1			19	14	5		
T staging				-0.166	0.305				0.207	0.188
T1	11	6	5			0	0	0		
T2	29	23	6			0	0	0		
T3	0	0	0			39	32	7		
T4	0	0	0			3	1	2		
N staging				-	-				-0.185	0.247
No	40	29	11			40	32	8		
N1	0	0	0			1	1	0		
Lost	0	0	0			1	0	1		
M staging				-	-				0.096	0.551
M0	40	29	11			40	32	8		
M1	0	0	0			1	1	0		
Lost	0	0	0			1	0	1		
Ctnm				-0.166	0.305				-0.064	0.692
Stage1	11	6	5			0	0	0		
Stage2	29	23	6			0	0	0		
Stage3	0	0	0			39	31	8		
Stage4	0	0	0			2	2	0		
Lost	0	0	0			1	0	1		
Ki67				-0.231	0.157				0.434	0.005
Ki67 low expression	25	17	8			28	25	3		
Ki67 high expression	14	11	3			13	7	6		
Lost	1	1	0			1	1	0		

old; tumor size was 1.5 cm to 14 cm; clinical stage distribution: stage1 in 11 cases, stage2 in 29 cases. 42 patients were included in the advanced HCC group (stage3/stage4). Among them, 39 males, 3 females; age distribution was ranged from 43 to 71 years; tumor size was 3 cm to 22 cm; clinical stage distribution: 39 cases in stage 3, 2 cases in stage 4, another 1 case can't be distinguished in stage3 or stage4.

Spearman correlation analysis showed that HDAC4 expression in cancerous had no relationship with clinical index in early HCC group. HDAC4 and Ki67 expression in cancerous had a positive relationship in advanced HCC group, but had no relationship with other clinical index. Detailed analysis results were shown in **Table 2**.

The correlation between HDAC4, ki67 expression and prognosis of HCC

Kaplan-Meier analysis and log-rank test were applied to determine the association between HDAC4, ki67 expression and prognosis of early

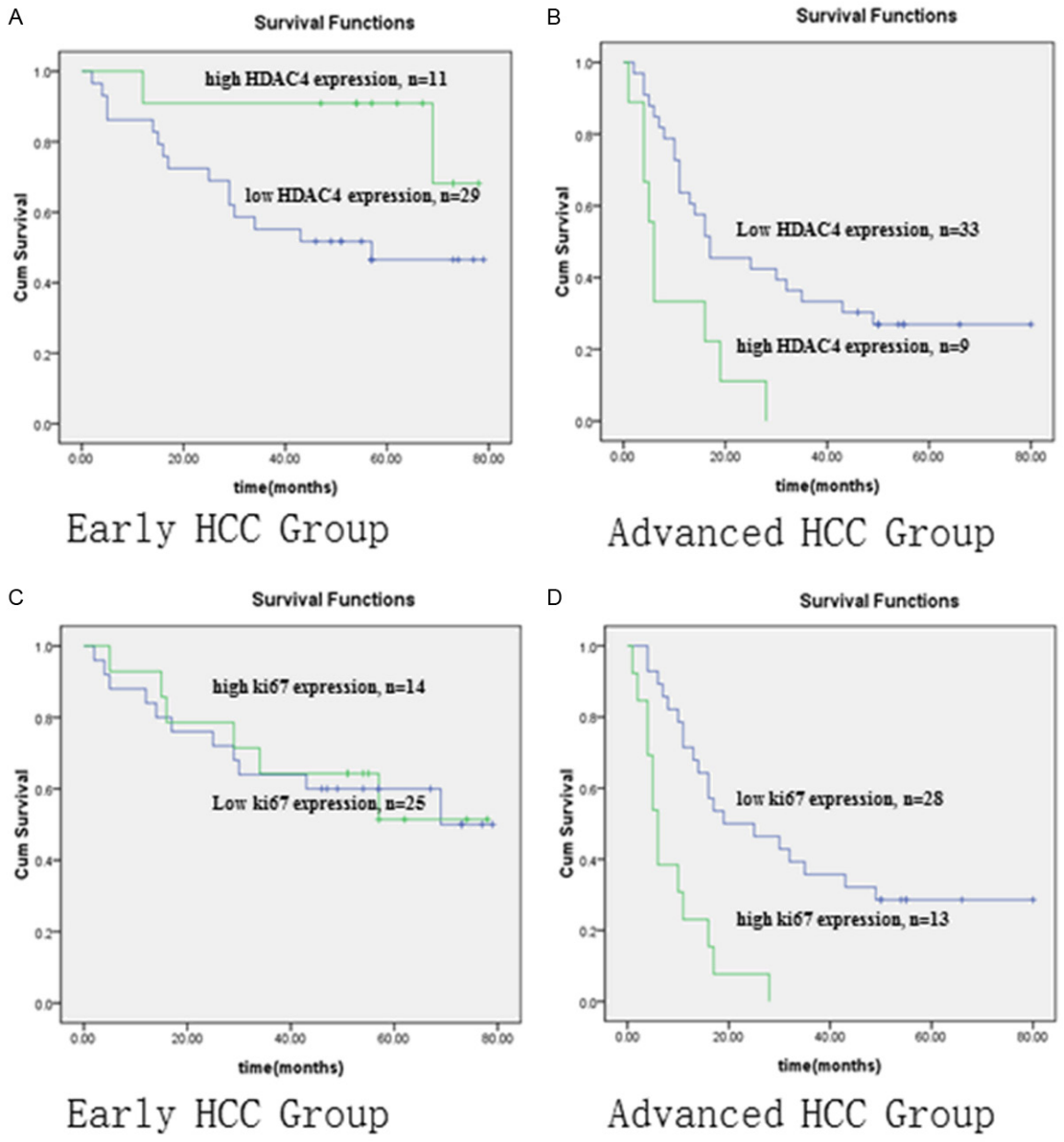

Figure 2. HDAC4 expression was positive correlated with the prognosis in early HCC group, but the *p* value had no significant difference (81.8% VS 48.3, P=0.057) (A); and the expression of ki67 had no relationship with prognosis (P=0.933) (C). However, the expression of HDAC4 and ki67 had significantly negative with the prognosis in advanced HCC group (0% VS 27.3, P=0.003; 0% VS 28.6%, P=0.000) (B and D).

HCC group and advanced HCC group respectively. The results showed HDAC4 expression was positive correlated with the prognosis in early HCC group, but the *p* value had no significant difference (81.8% VS 48.3, P=0.057); and the expression of ki67 had no relationship with prognosis (P=0.933). However, the expression of HDAC4 and ki67 had significantly negative with the prognosis in advanced HCC group (0% VS 27.3, P=0.003; 0% VS 28.6%, P=0.000). Detailed analysis was shown in **Figure 2**.

COX multi-factors analysis indicated that ki67 and M stage were independent predictors of advanced HCC group (P=0.046, P=0.028). Detailed analysis was shown in **Table 3**. There were no clinical index significantly correlated with prognosis in early HCC group (analyzed results were omitted).

Table 3. Analysis of independent prognostic factor in advanced HCC patients by Cox Multivariate analysis variables

	B	SE	Wald	df	*P*-value	Exp (B)	95.0% CI for Exp (B)	
							Lower	Upper
HDAC4 expression	.533	.565	.892	1	.345	1.705	.563	5.159
ki67 expression	1.053	.528	3.969	1	.046	2.866	1.017	8.073
M stage	3.230	1.471	4.823	1	.028	25.276	1.415	451.370

Discussion

As a member of the class II HDACs, HDAC4 showed certain caner promoting function in colon cancer, liver cancer and gastric cancer [6-8], but the correlation between it and the prognosis of hepatocellular carcinoma has not been reported. Therefore, we carefully chose a hepatocellular carcinoma tissue microarray which contained 90 cases with follow-up information, meanwhile, this experiment added ki67 clinical immunohistochemistry data, IHC technique and statistical analysis were applied to study the clinical significance of HDAC4 expression in the occurrence and development of HCC. Due to the occurrence and development of HCC related to the change of a series of gene network, the patients were divided into two groups according to the clinical stage and analyzed by statistical analysis. The results showed that: HDAC4 expression was significantly higher than para-cancerous tissue (P=0.001) in advanced hepatocellular carcinoma group (stage3/stage4); HDAC4 expression was significantly positively related to ki67 (r=0.434, P=0.005); and the two indicators were significantly negative related to prognosis respectively (0% VS 27.3%, P=0.003%; 0% VS 28.6%, P=0.000), Cox multivariate analysis showed that the expressions of ki67 and M staging is an independent predictor (P=0.046, P=0.028). In the early hepatocellular carcinoma group (stage1/stage2), the expression of HDAC4 was not related with ki67 expression (P=0.157), although HDAC4 expression in cancer tissues was still higher than the caner adjacent tissues (P=0.001); Patients with HDAC4 overexpression had better prognosis, although the *p* value was not significant (81.8% VS 48.3%, P=0.57). The results showed that HDAC4 might be regulated by different genes and perform different biological functions in different stages of hepatocellular carcinoma. In the later stage of hepatocellular carcinoma, HDAC4 might be involved in the ki67 signaling network and indirectly promote the proliferation of hepatocellular carcinoma cells and reduced the survival time of patients. But in the early stage of hepatocellular carcinoma, HDAC4 might participate an unknown tumor suppress network and prolong the survival time of patients. The tumor suppress function of HDAC4 was found in brain glioma by Cheng et al. [9]. They found that glioma patients with HDAC4 overexpression not only have better tumor grade, but also have significantly longer survival, and were more sensitive to chemotherapy and radiotherapy through analyzed CGGA, REMBERANDT, GSE1011 and GSE429-04 database's data. Further analysis also showed that HDAC4 expression has a significant negative correlation with chromosome instability (CIN). Therefore, they speculated that HDAC4 can improve the prognosis through maintaining the chromosome stable and inhibit the development of brain glioma. In conclusion, HDAC4 indeed has two sides of biological function [6-9]. Combined with the experimental results, we speculate that because of the complexity of the biological function of HDAC4 in hepatocellular carcinoma, it might not all HCC patients can benefit from HDAC4 inhibitors in the treatment.

At present, HDAC has become hot spot of tumor targeting therapy because the inhibitors of HDAC for most tumor cells have significant inhibitory effect [10, 11]. Among them, there are many reports about the inhibition of HDAC4 inhibitors on HCC cells. For example, Sun G et al. [12] found that valproic acid (VPA) can downregulate the expression of HDAC4 and upregulate acetylated histone 4 (AcH4), and inhibited growth and proliferation of hepatocellular carcinoma cell. At the same time, VPA can downregulate Notch1 and its target gene Hes1, and upregulted p21 and p63 expression. In vitro experiments, it showed that VPA combined with peptide drug CPT-SST (conjugate camptothecin-somatostatin) had a stronger inhibitory effect on hepatocellular carcinoma cell proliferation. In addition, some studies indicated

microRNA had the inhibitory effect on HDAC4. For example, Yuan JH et al. [13] found that miR-200a can bind with 3'UTR of HDAC4 and inhibited the expression of HDAC4, and subsequently facilitated the transcription of miR-200a, and upregulated the histone H3 acetylation of the promoter of miR-200a and p21, while increased the total content of acetyl-histone H3; conversely, HDAC4 can inhibit miR-200a expression and decrease the acetylation of histone H3 of miR-200a's promoter through Sp1 dependent pathway. Meanwhile, the vivo and vitro experiments showed that miR-200a can inhibit the proliferation and migration of hepatoma cells significantly.

Although the cytological experiments had confirmed that HDAC4 played a promoting role in hepatoma cell and HDAC4 inhibitor on HCC cells inhibition, however, our results showed HDAC4 can significantly reduce the survival time in patients with advanced hepatocellular carcinoma, but had no significant influence on the prognosis of early stage HCC. In the future, we plan to investigate more HCC samples and further study the correlation between HDAC4 and prognosis of different clinical stage. At the same time, in order to understand the HDAC4 inhibitors for the exact effect of HCC at different development stages, we will establish the model of human HCC in nude mice, and give HDAC4 inhibitor in different stage, and lay the foundation of clinical application in treatment of HCC.

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Disclosure of conflict of interest

None.

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