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Original Research

Clinical significance of HDAC9 in hepatocellular carcinoma

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Abstract: In recent years, most related studies have found that chronic hepatitis B virus infection is the main cause of hepatocellular carcinoma (HCC), but the specific pathogenesis is still unclear. To investigate the function of HDAC in hepatocellular carcinoma (HCC), this study used qRT-PCR to determine the expression levels of miR-376a and HDAC9 mNRA in HCC and para-cancerous tissues. The clinical significance of HDAC9 in HCC was assessed in a study cohort containing 37 patients with HCC using immunohistochemistry. The expression level of miR-376a in liver cancer tissues was significantly lower than that in para-cancerous tissues, while the expression level of HDAC9 mRNA in liver cancer tissue was significantly higher than that in para-cancerous tissues. The expression of HDAC9 occurred mainly in the nucleus. There was a significant correlation between tumor differentiation and HDAC9. Survival analysis showed that HCC patients with higher HDAC9 expression had poorer prognosis, and subsequent multivariate analysis showed that HDAC9 expression level was an independent predictor. There was a definite correlation between HDAC9 and the expressions of AFP and Ki67. These results suggest that the expression level of HDAC9 in HCC is abnormally high while the expression level of miR-376a is significantly decreased, indicating that HDAC9 may be a potential prognostic indicator of HCC.

Key words: Biological tissue microarray; Prognosis; Immunohistochemistry; Hepatocellular carcinoma; HDAC9; Ki67; AFP.

Introduction

Liver cancer is a serious threat to health, and its incidence and associated mortality are abnormally high in China (1). In recent years, most related studies have found that chronic hepatitis B virus infection is the main cause of hepatocellular carcinoma (HCC), but the specific pathogenesis is still unclear (2). At present, effective clinical treatment of HCC is hampered by the shortcomings associated with traditional radiotherapy and chemotherapy. These include side effects, poor efficacy, low surgical resection rate, high metastasis rate and high recurrence rate (3).

The development of tumors is a multi-factor, multi-stage process (4). Epigenetic modification abnormalities are one of the important features of tumors. Lysine acetylase is an important epigenetic modification in cells. Lysine deacetylases, often referred to as histone deacetylases (HDACs), are capable of removing histone and non-histone acetylation modifying groups, and they are involved in many physiological and pathological processes in cells (5). A total of 18 HDACs have been identified, of which HDAC9 is the most widely studied subtype (6-8). Studies have reported that HDAC2 promotes the development of HCC, and it can be used as a prognostic factor for HCC. At the same time, the expression level of HDAC9 is high in various tumor tissues and is closely related to the clinic-pathological features of patients (9, 10). Selective inhibitors of HDAC9 suppress the growth of a variety of tumor cells (11). However, the role of HDAC9 in HCC, especially for prognosis, is not fully understood.

MicroRNA (miRNA) has become one of the central issues in the field of tumor research in recent years. It is a small (about 18-25 nucleotides), single-stranded RNA molecule that does not encode a protein. As an oncogene or tumor suppressor gene, miRNA regulates the occurrence and development of tumor through interaction with downstream target genes (12). One of the miRNAs, MiR-376a is 20 to 24 nucleotides in length. It affects the expression level of genes by participating in post-transcriptional translation of regulatory genes. Recent studies have shown that miR-376a is up-regulated in esophageal cancer tissues and lung cancer tissues (13), and increased in plasma of breast cancer and ovarian cancer patients (14). Expression of miR-376a occurs in oxidized azomethane-induced colorectal cancer tissues (15). Its level of expression is closely related to the occurrence and development of CRC. However, there are few studies on the relationship between miR-376a and hepato-carcinogenesis, and some studies have confirmed that miR-376a directly regulates the expression of HDAC9. Therefore, the present study investigated the interaction between miR-376a and HDAC9 in liver cancer.

Materials and Methods

Subjects

In this study, the expression level of HDAC9 was determined using tissue microarrays (TMA, tissue chip technology) (HLiv-HCC180Sur-02) containing 37 cases of HCC tissues and 37 para-cancerous tissues (1.5 cm away from tumor). Thirty (30) of the HCC patients were male while 7 patients were female. Their ages were in the range of 28 – 78 years (median age = 59 years). Tumor sizes ranged from 1.3 to 1.4 cm. The clinical stages were: 5 cases in stage I, 13 cases in stage II, 16 cases in stage III, and 1 case in stage IV. Two of the cases did not receive clinical staging. The detailed clinical characteristics of the patients are shown in Table 2. Between January 2010 and December 2016, all patients diagnosed with hepatocellular carcinoma received no additional treatment prior to surgery and were followed up until September 2018. By the end of the follow-up period, a total of 24 patients died of HCC, with a median survival time of 15.7 months, while 13 patients were still alive. This study was approved the Ethics Committee of the Second People's Hospital of Wuhu, and was carried out in accordance with Declaration of Helsinki. Written informed consent was obtained from all subjected prior to tissue sampling.

qRT-PCR

Total RNA in tissues or cells was extracted according to the procedure in the TRIzol reagent kits (ThermoFisher, USA). The concentration and purity of each RNA extract were determined using an ultra-micro nucleic acid quantitative spectrometer (Thermo Nanodrop 1000, Thermo Scientific, USA). The reverse transcription operation was carried out according to the procedure on the miRNA reverse transcription kit (ThermoFisher, USA). Real-time PCR was performed according to the procedure in the miRNA qPCR kit instructions, and the relative expression level of miR-376a was calculated using $2^{-\Delta\Delta Ct}$ method, with U6 as an internal reference. The experiment was repeated 3 times independently. The primer sequences were:
miR-376a forward primer: 5'-GCTCTCTCGAGGCATTGCTAGAAATTGAGAAAACT-3, miR-376a reverse primer: 5'-GTCGAGCGGCCGCTGACTTTTCAAATTGTTAACTTTA-3'; HDAC9 forward primer: 5'- -GACATGGTCCTGGTTTCTGC-3', HDAC9 reverse primer: 5'- TCAAGAGCCAATGCCACAC-3'. U6 snRNA forward primer: 5'-CTCGCTTCGGCAGCACA-3', U6 snRNA reverse primer: 5'-AACGCTTCACGAATTGCGT-3'.

Immunohistochemical staining

Two-step immunohistochemistry was used to determine the expression of HDAC9, CD34, AFP and Ki67. In the first step, tissue sections were treated with EDTA buffer (acquired antigen), blocked with goat serum, and primary antibody (AbCAM, Ab109446, dilution ratio was 1:40,000; CD34, AbCAM, Ab8158, dilution ratio was 1:50; AFP, AbCAM, Ab46799, dilution ratio was 1:100; Ki67, AbCAM, Ab15580, dilution ratio was 1:500;) was applied, and the tissue sections were incubated overnight at 4 °C. In the second step, the sections were incubated with secondary antibody (DAKO, K8000), then washed with PBS, developed with diaminobenzidine (DAB) system, and counterstained with hematoxylin. Immunohistochemical staining intensity was assessed in the pathological sections. The scores were: negative = 0 points, + = 1 point, ++ = 2 points, and +++ = 3 points. A score of ≤ 1.5 was considered to be low expression, while scores > 1.5 were considered to be high expressions.

Statistical analysis

In this study, the NPAR method was used to evaluate differences in the expression levels of HDAC9 in hepatocellular carcinoma tissues and para-cancer tissues. Survival curves based on HDAC9 expression and clinical features were plotted using the Kaplan-Meier method and the log-rank test, and all potential predictors were then included in the Cox multivariate survival analysis. The correlation between HDAC9 mRNA and miR-376a expression was analyzed by Pearson correlation analysis. Spearman rank correlation coefficient was used to evaluate the correlation between HDAC9 expression level and clinical immunohistochemical factors such as CD34, AFP and Ki67 , as well as the correlation between HDAC9 expression in hepatocarcinoma tissues and para-cancer tissues. All statistical analyses were performed using SPSS 17.0 software. Values of $p < 0.05$ were considered statistically significant.

Results

Expression levels of miR-376a and HDAC9 mRNA in hepatocellular carcinoma tissues and para-cancerous tissues

Results from qRT-PCR showed that the expression level of miR-376a in hepatocellular carcinoma tissues (0.31±0.02) was significantly lower than that in para-cancerous tissues (1.26±0.11; $p < 0.05$; Figure 1A). The expression level of HDAC9 mRNA in hepatocellular carcinoma tissues (1.67±0.15) was significantly higher than that in para-cancerous tissues (0.54±0.04; $p < 0.05$; Figure 1B). Pearson correlation analysis showed a significant negative correlation between HDAC9 mRNA and miR-376a expressions (r = -0.356, $p < 0.05$; Figure 1C).

Expression level of HDAC9 in hepatocellular carcinoma tissues and para-cancerous tissues

As shown in Figure 2, HDAC9 was expressed in the nucleus of all tissue samples. However, the expression intensity of HDAC9 in HCC tissues was significantly higher than in the corresponding adjacent tissues (1.92±0.63 vs 1.08±0.48; $p < 0.05$; Table 1).

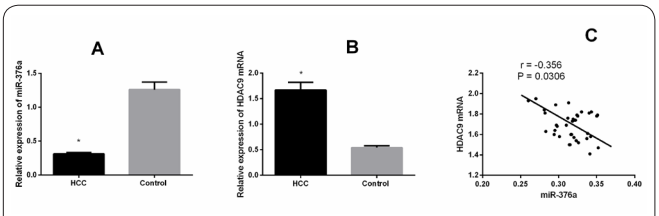

Figure 1. Expression levels of miR-376a and HDAC9 mRNA in HCC tissues and para-cancerous tissues, *$p < 0.05$, compared with the control group (para-cancerous tissues).

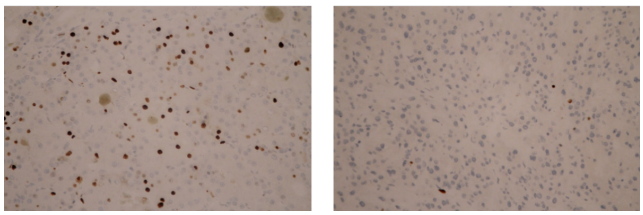

Figure 2. Immunohistochemistry of HDAC9: HDAC9 was present in the nucleus of all specimens. The staining intensity (H) in HDAC9 in HCC was higher than that in para-cancerous tissues (B) (magnification: x200).

Table 1. Expression of HDAC9 in HCC tissues and para-cancerous tissues.

Histology	n	Expression intensity	*p*
Hepatocellular carcinoma tissues	37	1.92±0.63	0.000
Para-cancerous tissues	37	1.08± 0.48	

The expression level of HDAC9 in HCC was positively correlated with the degree of tumor differentiation

As shown in Table 2, the expression level of HDAC9 in HCC tissues was significantly correlated with the degree of tumor differentiation ($r = 0.276$, $p < 0.05$), but there was no significant correlation with other clinical features ($p > 0.05$).

High level of expression of HDAC9 was associated with poor prognosis in patients with HCC

Survival analysis under different HDAC9 expressions is shown in Figure 3. The results showed that high level of HDAC9 expression was associated with poor prognosis in patients with HCC. The survival rate of patients with high HDAC9 expression was 18.5 %, while the survival rate of patients with low HDAC9 expression was 70.0 %. The effect of HDAC9 on cumulative survival was significant ($p < 0.05$). At the same time,

Table 2. Analysis of correlation between HDAC9 expression and clinical features of patients with HCC.

Clinical features	HDAC9 expression		Correlation coefficient	*p*
	Low (10 cases)	High (27 cases)		
Gender			0.059	0.548
Male	8	22		
Female	2	5		
Age			-0.193	0.068
≤60	7	20		
>60	3	6		
Lost	0	1		
Tumor size			-0.039	0.702
≤5cm	4	12		
>5cm	6	14		
Lost	0	1		
Tumor differentiation			0.276	0.007
I	0	0		
II	6	15		
III	4	12		
T staging			-0.077	0.448
T1	1	5		
T2	4	11		
T3	5	11		
T4	0	0		
Lost	0	0		
N staging			-0.169	0.121
N0	8	27		
N1	2	0		
Lost	0	0		
M staging			-0.005	0.969
M0	10	25		
M1	0	2		
Lost	0	0		
cTNM staging			-0.118	0.269
1	2	3		
2	5	10		
3	3	13		
4	0	1		
Lost	0	0		

Table 3. Univariate and multivariate analyses of clinical factors associated with HCC survival and HDAC9 expression.

Factor	Univariate	Multivariate		
	p	*p*	Exp (B)	95.0% CI for Exp (B)
Expression of HDAC9 in hepatocellular carcinoma	0.019	0.022	3.021	1.203-7.621
Age	0.347	——	——	——
Gender	0.741	——	——	——
Tumor size	0.011	0.351	1.388	0.711-2.773
Tumor differentiation	0.603	——	——	——
T staging	0.002	0.712	1.287	0.351-4.752
Staging	0.269	——	——	——
M staging	0.211	——	——	——
Clinical stage	0.002	0.602	1.379	0.421-4.496

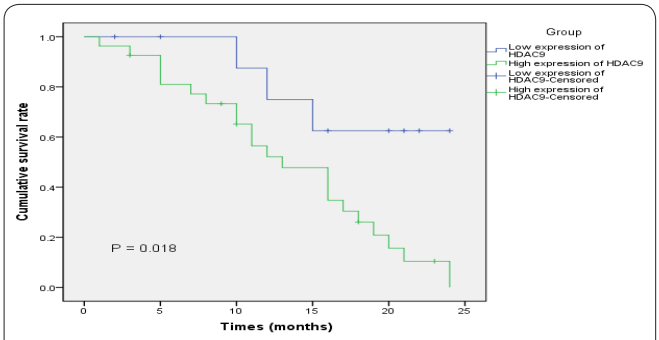

Figure 3. HCC survival analysis at different HDAC9 expression levels (P value using LOG-RANK test).

tumor size, T stage and clinical stage were associated with cumulative survival rate in HCC patients ($p < 0.05$). Multivariate analysis of clinical factors associated with HCC survival and HDAC9 expression indicated that HDAC9 expression was the only independent predictor ($p < 0.05$; Table 3).

Correlation between HDAC9 expression and expressions of AFP and Ki67 in HCC

To elucidate the regulatory mechanism of HDAC9 in the progression of HCC, Pearson correlation coefficient method was used to calculate the correlation between HDAC9 expression and CD34, and between AFP and Ki67 expressions. The results showed that the high level of HDAC9 expression was significantly correlated with high levels of AFP ($r = 0.379$, $p < 0.05$) and high expression of Ki67 ($r = 0.268$, $p < 0.05$). However, there was no significant correlation between HDAC9 expression level and CD34 expression level ($p > 0.05$). These results are shown in Figure 4 and Table 4.

Discussion

Chromatin acetylation plays an important role in regulating gene expression in eukaryotic cells and is regulated by two sets of opposite protein families, histone acetylase and histone deacetylase (HDACs). The formation of DNA chromatin complexes in eukaryotes requires the addition of core histones H2A, H2B, H3, and H4 to complete the organization and packaging of DNA. Thus, modification of core histones is important for changes in chromatin conformation. Histone acetylation is one of the important apparent modifications. The main function of HDACs is to remove the acetyl group from the N-terminus of acetyl lysine, so that the histone is positively charged and thus tightly bound to the negatively charged DNA. The staining exhibits a tightly curled tissue structure that inhibits transcription. Currently, 18 HDACs have been identified, and HDAC9 is one of the most studied in recent years. It has been confirmed to be involved in the occurrence and development of many of the common clinical cancers. Studies have shown that HDAC9 is abnormally expressed in many children with acute lymphoblastic leukemia, medulloblastoma, retinoblastoma, oral squamous cell carcinoma and osteosarcoma. In most cases, HDAC9 expres-

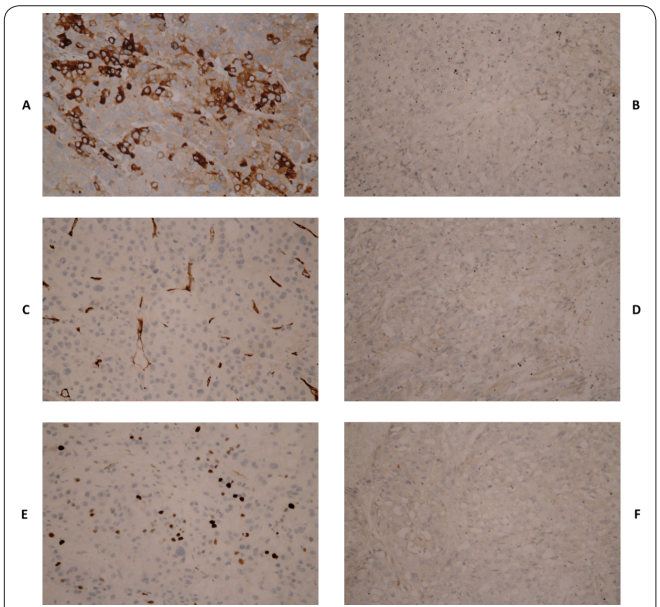

Figure 4. Expression of AFP in tumor tissue (A) and para-cancerous tissue (B), AFP was positively expressed in tumor tissue; expression of CD34 in tumor tissue (C) and para-cancerous tissue (D), CD34 was positively expressed in tumor tissue; expression of Ki67 in para-cancerous tissue (E) and tumor tissue (F) , but Ki67 was not positively expressed in both tissues.

Table 4. Correlation between HDAC9 expression and CD34, AFP and Ki67 expressions in HCC.

		CD34 expression	AFP expression	Ki67 expression
HDAC9 Expression	Correlation coefficient	-0.034	0.379	0.268
	Sig. (Two-tailed test)	0.768	0.001	0.012
	n	34	37	35

sion is up-regulated, and the results are comparable with previous findings, indicating consistency (6-7, 16-18). Although the molecular mechanism of HDAC9 in cancer is complex, most reports have confirmed the proliferative function of HDAC9. For example, it has been shown that HDAC9 inhibits apoptosis in tumor cells by inhibiting the expression of Microphthalmia-associated transcription factor (MITF) , and promotes the proliferation of tumor cells to local lymph nodes, thereby reducing the prognosis of patients with oral squamous cell carcinoma. In addition, although AFP has an independent prognostic role in HCC, it is also associated with HCC cell growth and Ki67 (19). Thus, it has been hypothesized that the high expression of HDAC9 may be a strong driving force for promoting the growth of HCC cells, which implies that it is associated with poor prognosis.

The carcinogenic effect of HDAC9 in HCC is not fully understood. However, because its expression level in HCC cells is negatively correlated with miR-376a, this may reflect the carcinogenic function of HDAC9 to some extent (20). The expression level of MiR-376a is down-regulated in HCC, while the high expression of MiR-376a plays a role in inhibiting tumor cell proliferation and inducing apoptosis of HCC tumor cells (21). Zheng and colleagues further confirmed that HDAC9 is a direct target of MiR-376a (20). Conversely, high expression of HDAC9 also inhibits the expression of MiR-376a. Therefore, overexpression of HDAC9 not only promotes the progression of HCC, but also inhibits apoptosis in HCC tumor cells.

In the present study, HDAC9 was specifically expressed in the nucleus of all sample tissues, and the expression level in HCC tissues was significantly higher than that in para-cancerous tissues. Analysis of the correlation between clinical features and HDAC9 expression showed that there was a significant correlation between tumor differentiation and HDAC9 expression levels. Survival analysis showed that HCC patients with high HDAC9 expression had a poor prognosis, and factor analysis showed that HDAC9 expression was the only independent predictor of HCC. To further understand the molecular mechanisms of HDAC9 in HCC, this study sought to explore the potential correlation between HDAC9 expression and clinical immunohistochemical factors (Ki67, AFP, and CD34). The results showed that there was a correlation between HDAC9 and AFP and Ki67: while the former was a poor prognostic factor for HCC, the latter was a marker for tumor cell proliferation. From the results obtained in this study, it can be speculated that HDAC9 plays a key role in HCC as an oncogene. The high expression of HDAC9 not only promoted the proliferation of tumor cells, but also hindered their differentiation, resulting in poor prognosis of liver cancer patients.

In addition, there was a certain correlation between AFP and HCC cell metastasis, and it affected HCC drug resistance after hepatectomy (22-24). Considering the relationship between HDAC9 expression and AFP expression, the potential effects of HDAC9 in HCC metastasis and drug resistance are of concern. The effect of HDAC9 expression on HCC cell metastasis and drug resistance needs further study.

This study has shown that miR-376a is down-regulated in HCC while HDAC9 is up-regulated, suggesting that HDAC9 may be a potential prognostic indicator of HCC. In subsequent studies, the molecular mechanism of HDAC9 will be investigated by knocking out or overexpressing HDAC9 in HCC cells so as to confirm if HDAC9 plays a role in promoting HCC.

Acknowledgements
None.

Conflict of Interest
There are no conflicts of interest in this study.

Author's contribution
All work was done by the author named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Jiefen Wu; Yuzhi Hu, Lei Sun, Shaofu Tao, Min Dai, Yandong Wang, Yong Li and Jiefen Wu collected and analysed the data; Yuzhi Hu wrote the text and all authors have read and approved the text prior to publication.

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