Abstract. – OBJECTIVES: Histone deacetylases (HDACs) play important roles in the regulation of genes expression and contribute to the growth of cancer cells. The present study aimed to investigate the function of HDAC5 in human hepatocellular carcinoma (HCC).

PATIENTS AND METHODS: The expression of HDAC5 in human hepatocellular carcinoma tissues and cells was detected. MTT assay was used to measure the proliferation of HCC cell lines. siRNA technology was employed to down-regulate the protein expression of HDAC5 and Six1.

RESULTS: Western blot showed that the HDAC5 expression was increased in human HCC tissues. The mRNA and protein levels of HDAC5 were up-regulated in human HCC cell lines. MTT assay showed that over-expression of HDAC5 promoted cell proliferation in human HCC cell lines. Down-regulation of HDAC5 caused a significantly inhibition of liver cancer cells proliferation. Furthermore, we found that HDAC5 promoted the Six1 expression both at the mRNA and protein levels in HCC cell lines.

CONCLUSIONS: The current study demonstrated for the first time that HDAC5 promoted HCC cell proliferation through up-regulation of Six1 expression and might provide novel therapeutic targets in the treatment of HCC.

Key Words: Hepatocellular carcinoma, Cell proliferation, HDAC5, Six1.

Introduction

Hepatocellular carcinoma (HCC) is one of the most malignant cancers, ranking the third cause of cancer-related mortality in the world. The current clinical treatments for HCC include radiotherapy, chemotherapy and surgical operation. However, increasing studies have shown that the resistance of HCC to conventional drugs has become a great challenge. Therefore, it is urgent to develop new and effective therapeutic methods for the treatment of HCC.

The histone deacetylase (HDACs) family contains a family of 18 proteins, which are classified into I, II, and IV based on their homology and structure. Classes I, II, and IV contain 11 family members, called classical HDACs, whereas the seven class III family members are referred to as sirtuins. HDAC5 belongs to the class II histone deacetylase family and has been shown to play critical roles in cell proliferation, cell cycle progression and apoptosis. Studies demonstrated that HDAC family members are involved in cancer initiation and metastasis. A recent study found that mRNA expression of HDAC5 was aberrantly expressed in HCC compared to the normal livers and suggested that the dysfunction of HDAC5 might play a significant role in hepatocarcinogenesis. However, the biological function of HDAC5 in human HCC has not been fully elucidated. Therefore, in the present study, we examined the expression of HDAC5 in human HCC samples and further investigate the biological function in HCC.

Patients and Methods

Clinical Samples
The samples from tumor tissues and adjacent non-tumor tissues were collected from eight three HCC patients undergone liver resection be-
tween Jan 2009 and June 2012. There were 56 men and 27 women, ranged from 33-74 years with a median age of 54 years. Eighteen normal liver tissues were recruited from donors free of liver diseases. All samples were obtained with informed consent from all the participants and this study was approved by the Institutional Review Board of Zhengzhou University and conducted according to the Declaration of Helsinki.

Cell Culture
Human HCC cell lines Hep3B and Huh7 and normal liver cell lines LO2 were obtained from Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), streptomycin (100 mg/mL) and penicillin (100 U/mL). Cultured cells were maintained at 37°C and 5% CO2 in a humid environment and passaged when the confluence reached 80%.

Plasmid Construction and Transfection
The cDNA fragment encoding HDAC5 was isolated using reverse transcriptase-polymerase chain reaction (RT-PCR) using total RNA from HCC cells. The primers sequences were as following: forward, 5’-GGA ATTCAT GAAGT TGAGG TGTTC GTC -3’ and reverse, 5’-CCT CAGCGCTACTCAGGCTAGGACGTCTC- CAC-3’.

The PCR product was cloned into the mammalian expression vector pcDNA3.1 (+) (Invitrogen, Carlsbad, CA, USA). siRNA-HDAC5 and siRNA Six 1 were purchased from Invitrogen (Carlsbad, CA, USA). Cells were transfected with lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the protocol.

Real-time PCR
Total RNAs were isolated from tissues or cells by TRIzol reagent, and reverse transcripts were performed by Takara RNA PCR kit (Takara, Bio Inc., Otsu, Shiga, Japan) following the manufacturer’s instructions. In order to quantify the transcripts of the interest genes, real-time PCR was performed using a SYBR Green Premix Ex Taq (Takara, Tokyo, Japan) on ABI 7500 system (Applied Biosystems, Foster City, CA, USA).

Western Blot Analysis
Cells were harvested by trypsinization, lysed in buffer and prepared for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After immunoblotting, the membranes were blocked in phosphate buffered saline (PBS)/0.1% Tween-20 with 5% nonfat dry milk, and primary antibodies were incubated in PBS/0.1% Tween-20 with 0.1%-5% nonfat dry milk. Antibodies directed against HDAC5, Six1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) with GAPDH used as a loading control.

 Statistical Analysis
All data were presented as mean ± SEM and treated for statistics analysis by SPSS program (SPSS Inc., Chicago, IL, USA). Comparison between groups were determined by ANOVA and statistical significance was displayed as *(p < 0.05) or **(p < 0.01).

Results
HDAC5 Expression was Increased in Human Hepatocellular Carcinoma Tissues and Cells
Real time PCR and western blot were used to examine the RNA and protein expression of HDAC5 from eight three pairs of tumor and non-tumor liver tissues. Results showed that the mRNA and protein expression were significantly increased in HCC tumor tissues compared with non-tumor tissues (Figure 1A and B). Furthermore, several HCC cell lines were analyzed by real time PCR and western blot. Data showed that higher expression of HDAC5 both at the mRNA and protein levels was observed in two HCC cell lines compared with normal liver cells (Figure 1C and D). Collectively, these data suggested that HDAC5 expression was up-regulated in HCC tissues and cell lines.

HDAC5 Overexpression Promoted the Proliferation of Hepatocellular Carcinoma Cells
We transfected HCC cell lines (Hep3B and Huh7) with plasmids encoding HDAC5 in order to investigate the function of HDAC5 in human HCC cells. MTT assay showed that HDAC5 over-expression significantly promoted the proliferation of Hep3B and Huh7 cells (Figure 2A and B). In addition, Hep3B cells were transfected with small interfering RNA (siRNA) targeting HDAC5. Two independent siRNA oligos showed
HDAC5 promotes HCC cell proliferation by up-regulating Six1

Efficient HDAC5 knockdown in U87 cells compared with scramble siRNA-transfected cells (Figure 2C). Results showed that HDAC5 down-regulation significantly inhibited the cell proliferation (Figure 2D). Similar results were also observed in Huh7 cells. Taken together, our results demonstrated that HDAC5 could promote the proliferation of hepatocellular carcinoma.

**HDAC5 Promotes Hepatocellular Carcinoma Proliferation by up-Regulation of Six1**

Next we explored the molecular mechanism underlying the proliferative effect of HDAC5, the results demonstrated that over-expression of HDAC5 significantly increased the Six1 protein expression (Figure 3A and B). Consistently, down-regulation of HDAC5 remarkably decreased the protein expression of Six1 (Figure 3C and D). Furthermore, the expression of Six1 was down-regulated using siRNA oligos as shown by real time PCR and western blot (Figure 4A and B). As a result, the decreased expression of Six1 blocked the effect of HDAC5 on cell proliferation in hepatocellular carcinoma (Figure 4C). Taken together, these results indicated that HDAC5 promoted cell proliferation by up-regulation of Six1.

**Discussion**

The main function of HDACs is to remove the acetyl groups from the N-acetylly-sines on histone and modify the chromatin structure, therefore, modulating the expression of many genes and biological processes\(^1\). The aberrant expression has shown to associated with tumor initiation and development\(^1\). The current study aimed to investigated the biological role of HDAC5 in human hepatocellular carcinoma tissues and cells lines. In our research, we found that both the
mRNA and protein expression of HDAC5 were significantly increased in hepatocellular carcinoma tissues and cells lines.

HDAC5, a class II histone deacetylase family member, has been demonstrated to be a critical regulator in cell apoptosis and proliferation in many cancer cell lines. A recent study found that HDAC5 and HDAC9 are significantly over-expressed in high-risk medulloblastoma in comparison with low-risk medulloblastoma, and their expression is associated with poor survival, suggesting that HDAC5 and HDAC9 may be important markers for risk stratification. Another research group demonstrated that HDAC5 could transport from nucleus to cytoplasm during the myoblasts differentiation and suppress the expression of a cell cycle activator Cyclin D3, implicating its function in cell differentiation and proliferation.

In our study, over-expression of HDAC5 in hepatocellular carcinoma significantly promoted the cell proliferation. Meanwhile, HDAC5 knockdown using siRNA suppressed the hepatocellular carcinoma cell proliferation, implicating the HDAC5 as a positive regulator in hepatocellular carcinoma proliferation.

Six1 belongs to a subfamily of the Six class of homeodomain-containing transcription factors, which share a lysine within the DNA-binding helix of the homeodomain. Studies have shown that over-expression of Six1 was found in a large percentage of breast cancer and strongly correlates with metastatic breast lesions. Six1 up-regulation in breast cancer significantly promotes tumor cell proliferation through activation of Cyclin A1. Moreover, Six1 plays a critical role in the regulation of the rhabdomyosarcoma metastatic ability. Our study found that HDAC5 significantly increased the expression of Six1. Furthermore, Six1 down-regulation diminished the proliferative effect of HDAC5 on hepatocellular carcinoma cells.
Figure 3. Effect of HDAC5 on Six1 expression in HCC cells. 

**A-B.** The protein expression of Six1 in Hep3B cells over-expressing HDAC5 was determined by western blot. Relative band intensities of each protein were quantified by densitometry. *p < 0.05; **p < 0.01.

**C-D.** The Six1 protein expression was measured by western blot in Hep3B cells over-expressing HDAC5. Relative band intensities of each protein were quantified by densitometry. *p < 0.05; **p < 0.01.

Figure 4. HDAC5 promotes HCC cell proliferation by up-regulation of Six1. 

**A-B.** Real-time PCR and western blot were performed to detect the mRNA and protein expression levels of Six1 in Hep3B cells transfected with Six1 siRNA oligos. *p < 0.05; **p < 0.01.

**C.** MTT assay was used to measure the cell proliferation in Hep3B cells over-expressing HDAC5 with or without Six1 knockdown using siRNA oligos.
The Authors declare that there are no conflicts of interest.

Conflict of Interest
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References