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HDAC5 promotes cell proliferation in human hepatocellular carcinoma by up-regulating Six1 expression

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Abstract. – OBJECTIVES: Histone deacetylases (HDACs) plays 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, Six 1.

Introduction

Hepatocellular carcinoma (HCC) is one of the most malignant cancers, ranking the third cause of cancer-related mortality in the world[1,2]. Statistical studies have shown that there is estimated 350 000 new cases and nearly one million deaths annually[3]. The current clinical treatments for HCC include radiotherapy, chemotherapy and surgical operation[4,5]. However, increasing studies have shown that the resistance of HCC to conventional drugs has becoming great challenges. Therefore, it is urgent to develop new and effective therapeutic methods for the treatment of HCC[6].

The histone deacetylase (HDACs) family contains a family of 18 proteins, which are classified into IñIV 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[7,8]. 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[9,10]. Studies demonstrated that HDAC family members are involved in cancer initiation and metastasis[11]. 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[12]. 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-

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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 confluency 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`-GGAATTCATGAAGTTGGAGGTGTTCGTC-3` and reverse, 5`-CCTCGAGCGCTACTCAGGCTAGGAGCGTCTCCAC-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 transcriptions were performed by Takara RNA PCR kit (Takara, Bio Inc., Otsu, Shiga, Japan) following the manufacturerís 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

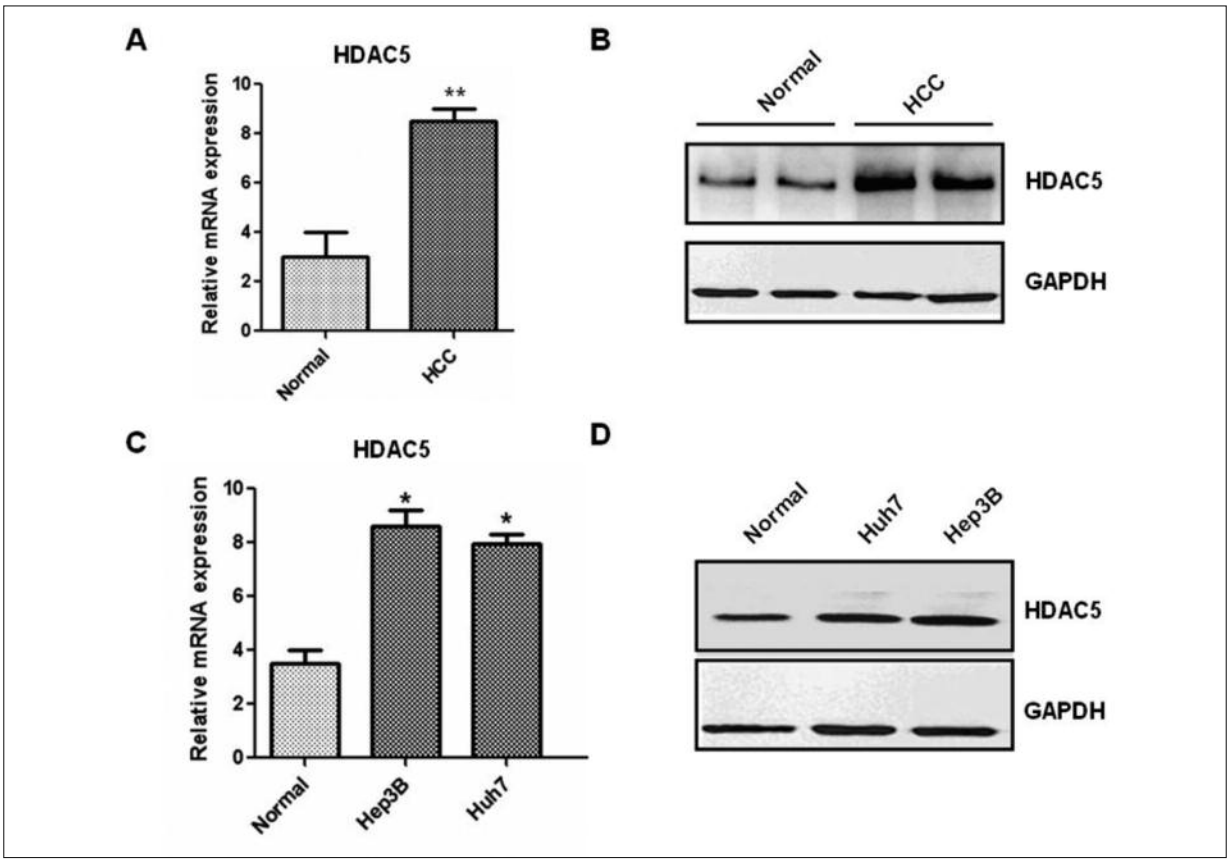

Figure 1. Up-regulation of HDAC5 in human hepatocellular carcinoma tissues and cell lines. ***A-B,*** The mRNA and protein levels of HDAC5 were determined by real-time PCR and western blot in human hepatocellular carcinoma tissues and normal tissues. *$p < 0.05$; **$p < 0.01$. ***C-D,*** Real-time PCR and western blot were performed to determine the HDAC5 expression in human normal liver cell lines LO2 and HCC cell lines including Hep3B and Huh7. *$p < 0.05$; **$p < 0.01$.

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[13]. The aberrant expression has shown to associated with tumor initiation and development[14]. 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

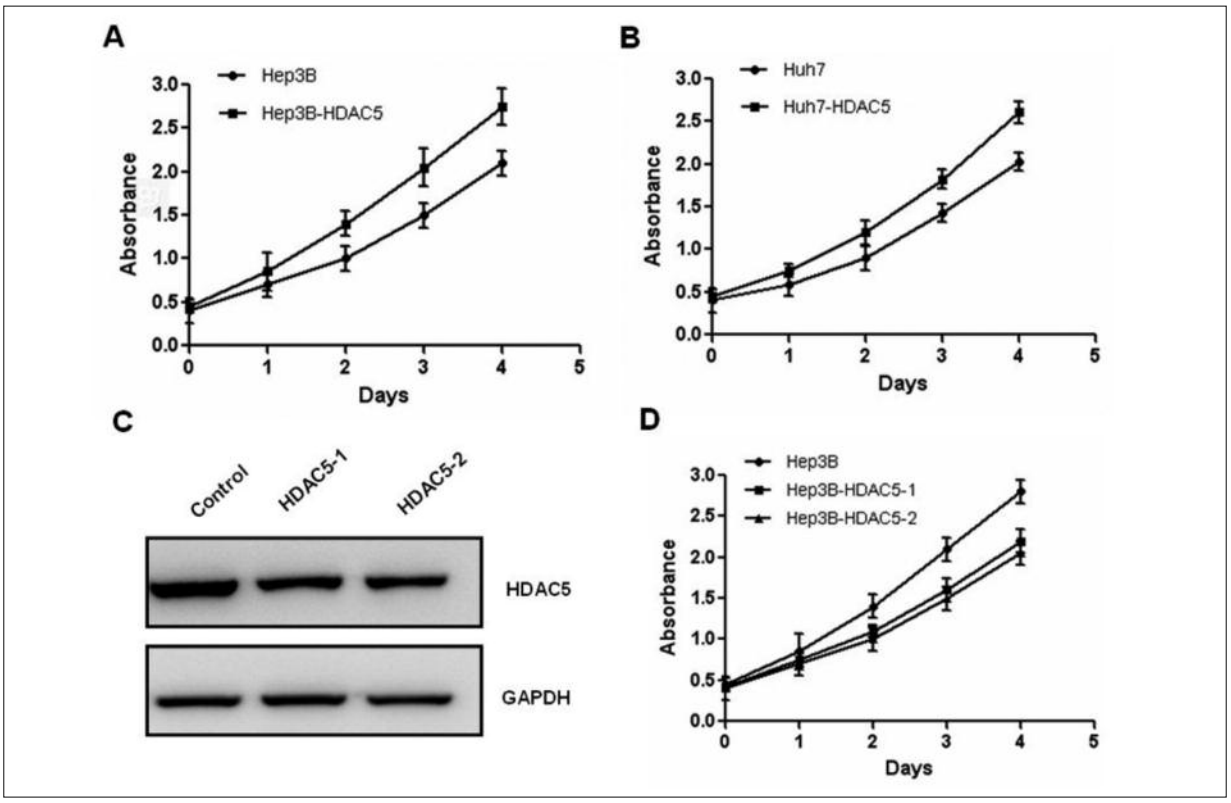

Figure 2. Effect of HDAC5 expression on the proliferation of hepatocellular carcinoma cells. The plasmids encoding HDAC5-flag were transfected into the HCC cell lines with lipofectamine 2000. Then, the proliferation of HCC cell line Hep3B ***(A)*** and Huh7 ***(B)*** was measured using the MTT assay. ***C,*** Hep3B cells were transfected with small interfering RNA (siRNA) targeting HDAC5. Western blot was used to analyze the down-regulation of HDAC5 in HCC cells after transfection with two independent siRNA oligos and scramble siRNA. ***D,*** The cells proliferation was measured using the MTT assay in Hep3B cells transfected with HDAC5 siRNA oligos.

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 an critical regulator in cell apoptosis and proliferation in many cancer cell lines[15]. 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[16]. 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[17,18].

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[19]. Studies have shown that over-expression of Six1 was found in a large percentage of breast cancer and strongly correlates with metastatic breast lesions[20]. Six1 up-regulation in breast cancer significantly promotes tumor cell proliferation through activation of Cyclin A1[21]. Moreover, Six1 plays a critical role in the regulation of the rhabdomyosarcoma metastatic ability[22]. 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.

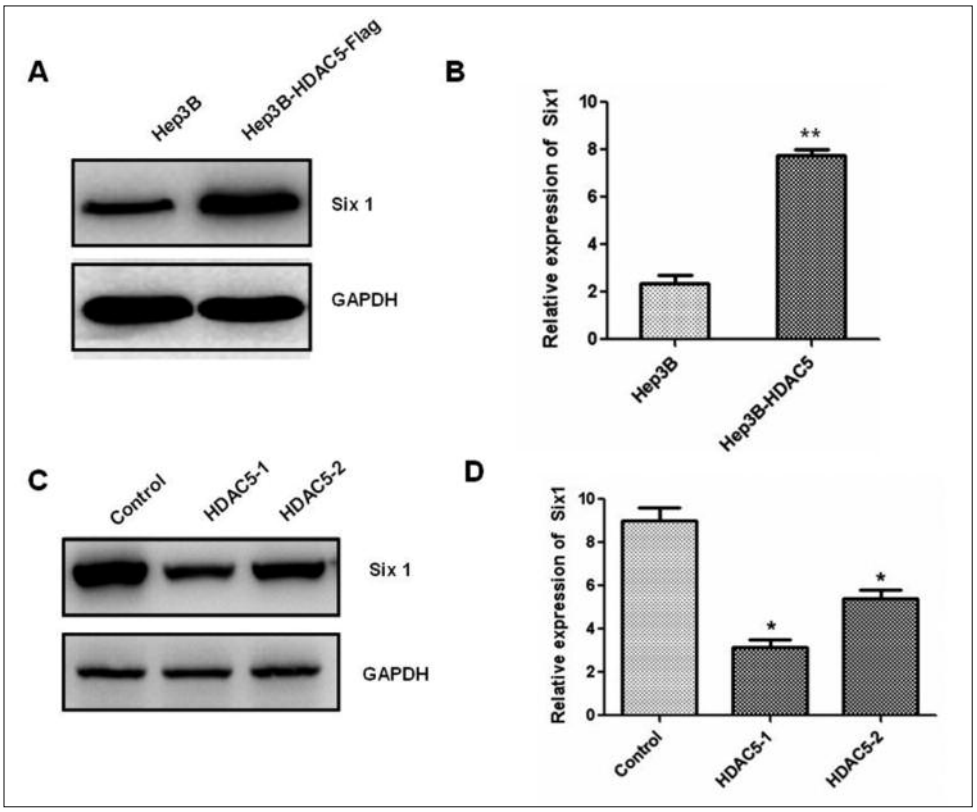

Figure 3. Effect of HDAC5 on Six1 expression in HCC cells. ***A-B,*** The protein expression of Six1 in Hep3B cells over-expressing HDAC5 was determined by western blot. Relative band intensities of each protein were quantified by densitometry. *$p < 0.05$; **$p < 0.01$. ***C-D,*** The Six1 protein expression was measured by western blot in Hep3B cells over-expressing HDAC5. Relative band intensities of each protein were quantified by densitometry. *$p < 0.05$; **$p < 0.01$.

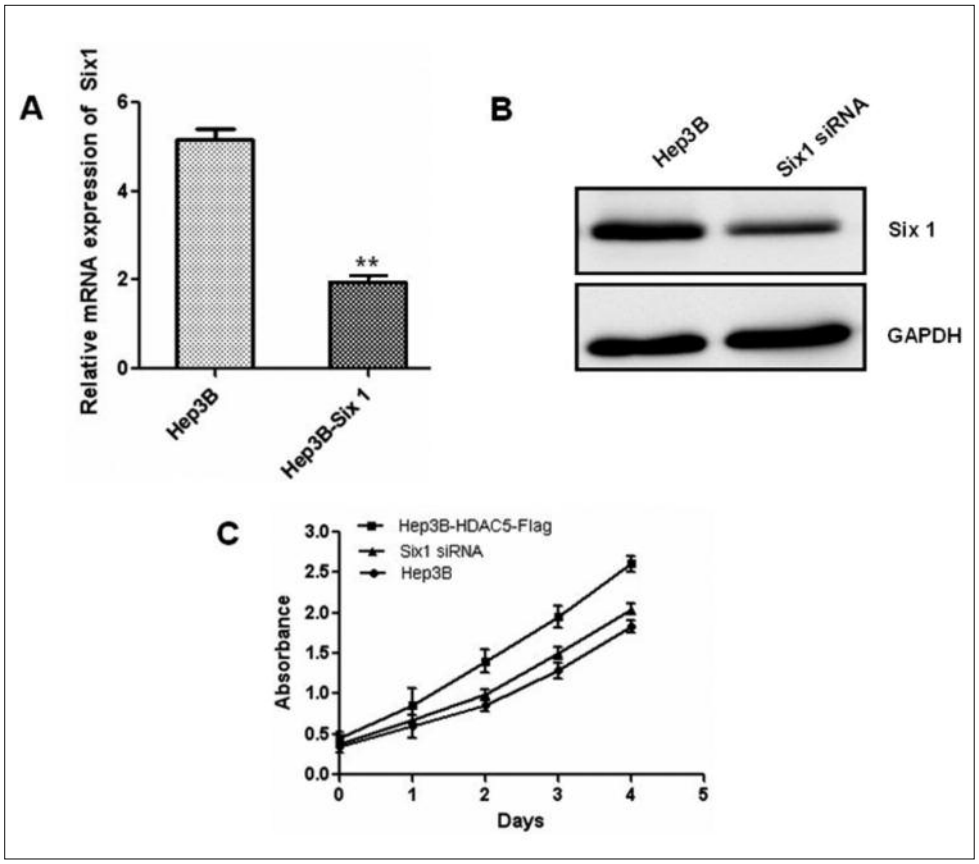

Figure 4. HDAC5 promotes HCC cells proliferation by up-regulation of Six1. ***A-B,*** Real time PCR and western blot were performed to detect the mRNA and protein expression levels of Six1 in Hep3B cells transfected with Six1 siRNA oligos. *$p < 0.05$; **$p < 0.01$. ***C,*** MTT assay was used to measure the cell proliferation in Hep3B cells over-expressing HDAC5 with or without Six1 knockdown using siRNA oligos.

Conclusions

The present work found that HDAC5 was significantly increased in the HCC samples and cell lines. Besides, our study further demonstrated that HDAC5 promotes the proliferation of HCC cells via up-regulation of Six1 and might provide novel therapeutic targets in the HCC treatment.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

References

1) El Serag HB. Hepatocellular carcinoma. N Engl J Med 2011; 365: 1118-1127.
2) Cabibbo G, Craxi A. Epidemiology, risk factors and surveillance of hepatocellular carcinoma. Eur Rev Med Pharmacol Sci 2010; 14: 352-355.
3) Tang ZY, Ye SL, Liu YK, Qin LX, Sun HC, Ye QH, Wang L, Zhou J, Qiu SJ, Li Y, Ji XN, Liu H, Xia JL, Wu ZQ, Fan J, Ma ZC, Zhou XD, Lin ZY, Liu KD. Decade's studies on metastasis of hepatocellular carcinoma. J Cancer Res Clin Oncol 2004; 130: 187-196.
4) Ursino S, Greco C, Cartei F, Colosimo C, Stefanelli A, Cacopardo B, Berretta M, Fiorica F. Radiotherapy and hepatocellular carcinoma: update and review of the literature. Eur Rev Med Pharmacol Sci 2012; 16: 1599-1604.
5) Minagawa M, Makuuchi M, Takayama T, Kokudo N. Selection criteria for repeat hepatectomy in patients with recurrent hepatocellular carcinoma. Ann Surg 2003; 238: 703-710.
6) Ling X, Wen L, Zhou Y. Role of mitochondrial translocation of telomerase in hepatocellular carcinoma cells with multidrug resistance. Int J Med Sci 2012; 9: 545-554.
7) West AC, Mattarollo SR, Shortt J, Cluse LA, Christiansen AJ, Smyth MJ, Johnstone RW. An intact immune system is required for the anticancer activities of histone deacetylase inhibitors. Cancer Res 2013; 73: 7265-7276.
8) Sebastian C, Zwaans BM, Silberman DM, Gymrek M, Goren A, Zhong L, Ram O, Truelove J, Guimaraes AR, Toiber D, Cosentino C, Greenson JK, MacDonald AI, McGlynn L, Maxwell F, Edwards J, Giacosa S, Guccione E, Weissleder R, Bernstein BE, Regev A, Shiels PG, Lombard DB, Mostoslavsky R. The histone deacetylase SIRT6 is a tumor suppressor that controls cancer metabolism. Cell 2012; 151: 1185-1199.
9) Wagner JM, Hackanson B, Lubbert M, Jung M. Histone deacetylase (HDAC) inhibitors in recent clinical trials for cancer therapy. Clin Epigenetics 2010; 1: 117-136.
10) Zhang Y, Matkovich SJ, Duan X, Diwan A, Kang MY, Dorn GN. Receptor-independent protein kinase C alpha (PKCalpha) signaling by calpain-generated free catalytic domains induces HDAC5 nuclear export and regulates cardiac transcription. J Biol Chem 2011; 286: 26943-26951.
11) Arts J, King P, Marien A, Floren W, Belien A, Janssen L, Pilatte I, Roux B, Decrane L, Gilissen R, Hickson I, Vreys V, Cox E, Bol K, Talloen W, Goris I, Andries L, Du Jardin M, Janicot M, Page M, van Emelen K, Angibaud P. JNJ-26481585, a novel "second-generation" oral histone deacetylase inhibitor, shows broad-spectrum preclinical antitumoral activity. Clin Cancer Res 2009; 15: 6841-6851.
12) Lachenmayer A, Toffanin S, Cabellos L, Alsinet C, Hoshida Y, Villanueva A, Minguez B, Tsai HW, Ward SC, Thung S, Friedman SL, Llovet JM. Combination therapy for hepatocellular carcinoma: additive preclinical efficacy of the HDAC inhibitor panobinostat with sorafenib. J Hepatol 2012; 56: 1343-1350.
13) Rincon-Arano H, Halow J, Delrow JJ, Parkhurst SM, Groudine M. UpSET recruits HDAC complexes and restricts chromatin accessibility and acetylation at promoter regions. Cell 2012; 151: 1214-1228.
14) Lehmann A, Denkert C, Budczies J, Buckendahl AC, Darb-Esfahani S, Noske A, Muller BM, Bahra M, Neuhaus P, Dietel M, Kristiansen G, Weichert W. High class I HDAC activity and expression are associated with RelA/p65 activation in pancreatic cancer in vitro and in vivo. BMC Cancer 2009; 9: 395-398.
15) Lee CK, Wang S, Huang X, Ryder J, Liu B. HDAC inhibition synergistically enhances alkylator-induced DNA damage responses and apoptosis in multiple myeloma cells. Cancer Lett 2010; 296: 233-240.
16) Milde T, Oehme I, Korshunov A, Kopp-Schneider A, Remke M, Northcott P, Deubzer HE, Lodrini M, Taylor MD, von Deimling A, Pfister S, Witt O. HDAC5 and HDAC9 in medulloblastoma: novel markers for risk stratification and role in tumor cell growth. Clin Cancer Res 2010; 16: 3240-3252.
17) Roy S, Shor AC, Bagui TK, Seto E, Pledger WJ. Histone deacetylase 5 represses the transcription of cyclin D3. J Cell Biochem 2008; 104: 2143-2154.
18) McKinsey TA, Zhang CL, Lu J, Olson EN. Signal-dependent nuclear export of a histone deacetylase regulates muscle differentiation. Nature 2000; 408: 106-111.
19) Oliver G, Wehr R, Jenkins NA, Copeland NG, Cheyette BN, Hartenstein V, Zipursky SL, Gruss P. Homeobox genes and connective tissue patterning. Development 1995; 121: 693-705.
20) Reichenberger KJ, Coletta RD, Schulte AP, Varella-Garcia M, Ford HL. Gene amplification is a mechanism of Six1 overexpression in breast cancer. Cancer Res 2005; 65: 2668-2675.
21) Coletta RD, Christensen K, Reichenberger KJ, Lamb J, Micomonaco D, Huang L, Wolf DM, Muller-Tidow C, Golub TR, Kawakami K, Ford HL. The Six1 homeoprotein stimulates tumorigenesis by reactivation of cyclin A1. Proc Natl Acad Sci U S A 2004; 101: 6478-6483.
22) Yu Y, Khan J, Khanna C, Helman L, Meltzer PS, Merlino G. Expression profiling identifies the cytoskeletal organizer ezrin and the developmental homeoprotein Six-1 as key metastatic regulators. Nat Med 2004; 10: 175-181.