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Histone deacetylase 3 participates in self-renewal of liver cancer stem cells through histone modification

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ABSTRACT

Understanding molecular mechanisms in self-renewal of cancer stem cells (CSCs) is important for finding novel target in therapy of cancer. In this study, we explored potential effects of histone deacetylase (HDAC) on liver CSCs. Our data showed that HDAC inhibitors suppressed self-renewal and induced differentiation of liver CSCs. Furthermore, we demonstrated that HDAC3 was selectively expressed in liver CSCs and participated in self-renewal of liver CSCs via regulating expression of pluripotency factors. Overexpression of HDAC3 was associated with poor outcome of liver cancer. HDAC inhibitors could render liver CSCs sensitive to sorafenib. Taken together, our data suggest that HDAC3 plays a critical role in regulating self-renewal of liver CSCs.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most frequent malignancies with high mortality rate [1]. A recent concept about tumorigenesis is cancer stem cells (CSCs). In the CSCs model, there is a small subset of CSCs which constitute a reservoir of self-sustaining cells with the exclusive ability to self-renew and maintain the tumor. These CSCs have the capacity of expanding the CSCs pool and differentiating into the heterogeneous non-tumorigenic cancer cell types that in most cases appear to constitute the bulk of the cancer cells within the tumor [2]. It has been shown that high-grade tumors are enriched with a high content of CSCs [3]. In liver cancer, CSCs have been isolated by several cell surface antigens such as CD133 [4], CD90 [5], EpCAM [6], CD13 [7] and CD24 [8]. It has been demonstrated that these CSCs are responsible for tumor initiation, resistance to therapeutic regimens and tumor recurrence after surgical removal of primary tumors [2,9,10]. CD24 molecule can drive self-renewal and tumor initiation of liver CSCs though STAT3-mediated Nanog regulation [8]. Our previous study has identified transcription factor Nanog as a novel marker for liver CSCs and demonstrated that Nanog plays an important role in regulating self-renewal of liver CSCs via IGF signaling pathway [11]. However, the key components and molecular mechanisms contributing to self-renewal of CSCs are largely unknown.

Previous studies have indicated that chromatin structure including histone modifications and DNA methylation are features of cancers, early embryonic development, and differentiation of stem cells [12–16]. Histone acetylation is a dynamic process resulting from the balance between histone acetyltransferases and histone deacetylases (HDACs) [16]. Carcinogenesis is associated with a relative decrease in histone acetylation by numerous molecular mechanisms [16]. It has been reported that the altered expression of HDACs is associated with a number of human cancers including HCC and HDACs play a critical role in the development of cancers [17–20]. Epigenetic regulation by histone lysine acetylation plays a key role in regulating self-renewal capability of embryonic and adult stem cells [12,21]. HDAC inhibitors can induce inhibition of proliferation and induction of apoptosis in many types of cancer cell [22,23]. Some HDAC inhibitors are being tested clinically as anticancer agents for the treatment of leukemia and a variety of solid tumors [24]. However, little is known about the function of HDAC inhibitors on liver CSCs and which member of HDAC family to regulate self-renewal of liver CSCs.

In this study, we investigated the effect of HDAC inhibitors on liver CSCs. We found that HDAC3 participated in the self-renewal

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of liver CSCs through histone modification and HDACs inhibitors rendered liver CSCs sensitive to the therapy of sorafenib.

2. Materials and methods

2.1. Tissue samples and cell lines

Fresh and paraffin fixed tumor specimens were obtained with informed consent from all patients according to the protocols approved by the Institutional Review Board of the Southwest Hospital, Third Military Medical University. All patients underwent surgical resection of primary HCC at the Institute of Hepatobiliary Surgery, Southwest Hospital, Third Military Medical University. The human HCC cell lines (Huh7 and PLC/PRF/5) were purchased from Shanghai Cell Collection (Shanghai, China). Patient-derived primary HCC cultures of tumor cells (T1115 and T1224) were prepared in our laboratory as described previously [11]. All cells were cultured in DMEM (Gibco) medium with 10% FBS (Gibco) at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2. Cell sorting by flow cytometry

HCC cell lines and patient-derived primary HCC cultures of tumor cells were infected with Lv-P_{Nanog}-GFP at MOI of 10 as described previously [11]. GFP expression in Lv-P_{Nanog}-GFP infected cells was analyzed by flow cytometry. Dead cells were excluded by 7-AAD staining. Top high (<5%) and top low (<5%) were chosen for sorting $Nanog^{Pos}$ and $Nanog^{Neg}$ cells. To obtain CD133+ cells, HCC cells were marked with PE-labeled anti-CD133 antibody (Miltenyi Biotec) at 4 °C for 15 min. Top high (<5%) and top low (<5%) were chosen for sorting CD133+ and CD133− cells. The stained cells were analyzed and sorted with FACS Aria II (BD Biosciences). Purity of the sorted cells was over 99%.

2.3. Sphere formation assay

A total of 200 the sorted $Nanog^{Pos}$, $Nanog^{Neg}$ or $CD133^{Pos}$, $CD133^{Neg}$ cells were plated into Costar® Ultra Low Cluster 24-well plates (Corning). The cells were cultured in the DMEM/F12 medium (Sigma) supplemented with B27 (Gibco), antibiotics, 20 ng/mL EGF, 20 ng/mL bFGF (Peprotech) and 10 ng/mL HGF (Peprotech) in the absence or presence of different concentrations of HDAC inhibitors, and 1% methyl cellulose was added to prevent cell aggregation. Cells were incubated at 37 °C for 14 days and numbers of spheres were counted.

2.4. Colony formation assays

Briefly, 1×10^4 cells were seeded in 10-cm tissue culture plates or 200 cells were seeded in 24 well plates. The cells were cultured in the DMEM medium supplemented with 10% of FBS in the absence or presence of different concentrations of HDAC inhibitors for 14 days. The colonies were fixed with 4% formaldehyde and stained with 0.1% crystal violet (Sigma–Aldrich). Numbers of clones were counted.

2.5. Tumor formation assay

SCID mice at age of 3–5 weeks, male, were maintained in pathogen-free conditions at animal facility of Third Military Medical University and received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences. The different numbers of $Nanog^{Pos}$ after treatment with HDAC3 siRNA or scramble siRNA were resuspended in serum-free medium and mixed with Matrigel at the ratio of 1:1. The cells were subcutaneously injected into SCID mice. Tumor formation was evaluated regularly after injection by palpation of injection sites for 3 months.

2.6. Induction of HCC with diethylnitrosamine (DEN)

Male Wistar rats (6 weeks old, 170 g) were maintained in pathogen-free conditions at the animal facility of Third Military Medical University and received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences. An acclimatization period of 4 days was carried out. The weight of the rats was recorded every week. Animals received 10 mg/kg/day of DEN (Sigma) for 24 weeks. Rats were given the weekly dose of DEN in drinking water (0.01% v/v) corresponding to the estimated water consumption of 6 days. Once the animals consumed the administered DEN solution, they were given DEN-free water for the rest of the week. DENA solution was prepared each week.

2.7. Immunohistochemical staining

Tissue specimens were obtained with informed consent from 76 patients who underwent hepatectomy for HCC at the Institute of Hepatobiliary Surgery, Southwest Hospital, Third Military Medical University, China. A tissue array block containing both HCC and non-HCC samples from these patients was constructed. Immunohistochemical staining was performed as previously described [11]. The clinical and pathological characteristics of the patients were summarized (Supplementary Table 1).

2.8. Cell proliferation assay

Cell proliferation was assessed by using CCK-8 assay according to the manufacturers' recommendations. Briefly, cells were plated at 1000 cells/well on a 96-well plate. The cells were treated with HDAC inhibitors at different concentrations in DMEM medium with 10% of FBS and the cell survival was measured at different time points after treatment.

2.9. Reverse transcription PCR analysis

Total RNA was extracted from the cells with RNAiso (Takara) according to the manufacturer's protocol. For mRNAs detection, reverse transcription was performed according to the protocol of RevertAid™ First Strand cDNA Synthesis Kits (Fermentas); qPCR was performed with SYBR premix Ex Taq (TaKaRa) on an Applied Biosystems 7300 Real Time PCR System supplied with analytical software (Applied Biosystems, USA). GAPDH mRNA was used to normalize RNA inputs. Primers for markers of stem cells and mature hepatocytes were shown at Supplementary Table 2. Data were processed by using the $2^{-\Delta\Delta Ct}$ method. The results were represented as the means ± SD of three independent experiments.

2.10. Immunofluorescence analysis

Cells were grown on glass cover-slips in a six-well plate and washed three times with PBS before fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in PBS for 5 min at room temperature (RT). Cells were blocked with 10% FBS (Gibco) in PBS for 30 min at RT. Cover-slips were then incubated with respective primary antibodies (Supplementary Table 3). Secondary antibodies were donkey anti-rabbit IgG-Alexa Fluor 647, or donkey anti-mouse IgG-Alexa Fluor 647 (Invitrogen). Cells were further washed in PBS and mounted with vectashield mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI) for counterstaining nuclei. Cells were analyzed by using fluorescence microscopy.

2.11. Western blot analysis

Cells were harvested and lysed in the lysis buffer for 30 min at 4 °C. Total cell extracts were separated in 12% SDS–polyacrylamide gel electrophoresis (PAGE) and then transferred on PVDF membrane (Millipore). The membrane were then blocked in 5% milk for 2 h at RT and blotted with antibody overnight at 4 °C. Antibodies used in the study were listed in Supplementary Table 3. After washing with phosphate buffered saline with Tween-20 (PBST) and incubating with either anti-rabbit IgG or anti-mouse IgG horseradish peroxidase-conjugated secondary antibody at a dilution of 1:2000 in PBST, immunocomplexes were visualized by using SuperSignal West Femto Chemiluminescent Substrate (Pierce). For quantification, signals were densitometrically normalized to GAPDH by GeneTools image analysis program (SynGene).

2.12. Statistical analysis

A Student's *t* test was used to calculate the statistical significance of the experimental data. The Kaplan–Meier survival curves and log-rank test were used for estimation of survival and difference between groups. The level of significance was set as $^{*}P < 0.05$ and $^{**}P < 0.01$. All data were presented as the means ± standard deviation (SD). The software tools SPSS 10.0 and Microsoft Excel were used.

3. Results

3.1. Inhibition of cell growth and self-renewal of liver CSCs by HDAC inhibitors

To understand whether the altered HDAC activity regulated cell growth and self-renewal of liver CSCs, we tested the effect of HDAC inhibitors (TSA and SAHA) on the liver CSCs isolated from HCC cells either by CD133 biomarker or by our newly established method to obtain pluripotency transcription factor Nanog-positive liver CSCs [11]. Our results showed that TSA could inhibit cell growth of $Nanog^{Pos}$ or $CD133^{Pos}$ cells isolated from HCC cell line Huh7 and patient-derived primary HCC cultures of tumor cells T115 in dose- and time-dependent manners (Fig. 1A and B, and Supplementary Fig. 1A). Similarly, TSA could also inhibit cell growth of $Nanog^{Neg}$ cells from Huh7 cell line (Supplementary Fig. 2A). There was higher expression of proliferation marker Ki-67 in $Nanog^{Pos}$ cells than