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Original Article

Silencing of the HDAC1 by siRNA inhibits proliferation and invasion of hepatocellular carcinoma cells

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Abstract: Background: HDAC1 plays an important role in a variety of cancers such as hepatocellular carcinoma. The present study aims to investigate effect of siRNA-HDAC1 on expression of HDAC1 and biological behavior of hepatoma cells. Methods: Protein expression of HDAC1 in hepatoma cells were determined by Western blot. Effects of siRNA-HDAC1 on migration and invasion of hepatoma cells were determined by using wound healing and transwell assays. Effects of siRNA-HDAC1 on cell-cycle related proteins including Cyclin D1, p21 and p27 were analyzed by using Western blot. Additionally, expressions of Zonula occludens-1 (ZO-1), E-cadherin and vimentin were measured by using Western blot. Immunofluorescence was used to observe morphology of nucleus. Flow cytometry was employed to detect cell apoptosis. Results: Expression of HDAC1 in HCC cell lines was significantly increased in comparison to LO2 cell line ($p < 0.01$). Additionally, Reverse transcription polymerase chain reaction (qPCR) and Western blot analyses revealed that expression of HDAC1 was significantly decreased after transfection with siRNA-HDAC1. Protein expression of CyclinD1 was decreased ($p < 0.01$) and protein expressions of p21 and p27 were increased ($p < 0.01$) in HCCLM3 cells transfected with siRNA-HDAC1. Additionally, siRNA-HDAC1 significantly increased protein expressions of E-cadherin and ZO-1 ($p < 0.01$), and significantly decreased protein expression of vimentin in HCCLM3 cells. Moreover, flow cytometric analysis showed that siRNA-HDAC1 transfection induced apoptosis of HCCLM3 cells ($p < 0.01$). Conclusion: siRNA-HDAC1 transfection affects proliferation, invasion, and metastasis of HCCLM3 cells. The mechanism of siRNA-HDAC1 is related to regulation cell-cycle related proteins and EMT related proteins.

Keywords: HDAC1, hepatocellular carcinoma, cell biological behavior

Introduction

The occurrence and development of hepatocellular carcinoma (HCC) is a complex process, which is related to a series of physiological changes, including apoptosis, cell proliferation, metastasis and invasion. Some symptoms such as loss of appetite, weight loss, and perforation are accompanied with patients who have hepatocellular carcinoma. Intracellular acetylation and deacetylation levels are in a dynamic equilibrium under the normal physiological conditions. However, in tumor microenvironment, activated histone deacetylase 1 (HDAC1) is recruited in a specific promoter region and further inhibits transcription of genes in relevant tumor proliferation, invasion, and metastasis [1]. Histone deacetylases (HDACs) are enzymes involved in expression of DNA, and are classified in four classes. Within the Class I HDACs, HDAC 1, 2, and 3 are found primarily in the nucleus, whereas HDAC8 is found in both the nucleus and the cytoplasm. Taunton and colleagues identified that HDAC1 belonging to mammal HDACs family as early as 1996. As an important member of HDACs family, altered expression and mutations of HDAC1 have been linked to tumor development since it induces the aberrant transcription of key genes regulating cellular functions such as cell proliferation, cell-cycle and apoptosis.

Senese and colleagues demonstrated that in the absence of HDAC1 cells arrest either at the G1 phase of the cell cycle or at the G2/M transition, resulting in the loss of mitotic cells, cell growth inhibition, and induction of cell apoptosis [2]. Additionally, they find that inactivation of

HDAC1 affects the transcription of specific target genes involved in cell proliferation and apoptosis. Cyclin D1 is a key protein required for progression through the G1 phase of the cell cycle. During the G1 phase, it is synthesized rapidly and accumulates in the nucleus, and is degraded as the cell enters the S phase. Cyclin D1 dimerizes with CDK4/6 to regulate the G1/S phase transition and entry into the S-phase. In addition, p21 and p27 are also known as cyclin-dependent kinase inhibitors, which can inhibit cyclin-CDK complexes [3, 4]. p21 is encoded by the *CDKN1A* gene located on chromosome 6 in human, and inhibits the activity of cyclin-CDK complexes. The p21 blocks interaction with CDK and cyclins, further preventing CDK activation. p21 is located on chromosome 12 in human, which prevents the activation of cyclin E-CDK2 complex, and thus controls the cell cycle progression at G1 phase. Many researches demonstrate an inverse correlation with p21and p27 expressions and the proliferation and metastasis of HCC, implying the prognostic value of p21and p27 [5, 6].

The epithelial-mesenchymal transition (EMT) is a complex process by which epithelial cells lose their cell polarity and cell-cell adhesion, and gains migratory and invasive properties to become mesenchymal stem cells. Numerous researches demonstrate that EMT plays an important role in the occurrence and development of cancers and is a key factor in the initiation of metastasis for cancer progression [7]. Vimentin is an intermediate filament protein that is highly expressed in mesenchymal tissue. It is known that the increasing level of vimentin promotes invasion and metastasis of cancers, indicating that downregulation of vimentin significantly inhibits invasion and metastasis of cancers. Zonula occludens-1 (ZO-1), also known as tight junction protein-1, has a role as a scaffold protein. Abnormal function of ZO-1 is related to occurrence of a variety of diseases. In addition, E-cadherin is a biomarker in epithelial cells and the absence of E-cadherin is associated with tumor invasion, migration and poor prognosis [8].

Materials and methods

Cell lines and reagents

The hepatoma cell lines including HepG2, HCCLM3 and SSMC-7721, and human fetal hepatocyte line were purchased from the Cell Bank of the Chinese Academic of Sciences. 3- [4, 5-Dime-thylthylthiazol-2-yl] -2, 5-diphenyltetrazolium bromide (MTT) and crystal Violet were purchased from Sigma Chemical Co. Rabbit polyclonal antibodies against CyclinD1, p21, p27 and ZO-1 were purchased from Epitmics Co. Rabbit polyclonal antibodies against Vimentin and ZO-1 were purchase from Abcam Co. Cell cycle analysis kit was purchased from Beyotime Institute of Biotechnology. Transwell chamber was purchased from Corning Co. Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM) and trypsin were purchased from Gibco Com. A HDAC1 siRNA and a scrambled siRNA were purchased from Shanghai GenePharma Co. The sense and antisense sequences of HDAC1 siRNA were 5'-GCC GGU CAU GUC CAA AGU ATT-3', and 5'-UAC UUU GGA CAU GAC CGG CTT-3'. The sense and antisense sequences of scrambled siRNA were 5'-GCC CTG AGG GCC CGA ACT GTT A-3', and 5'-CAG ACG CAC GGC TTT GAC CTT C-3'.

CO_2 Incubators and biosafety cabinet were purchased from Thermo Scientific Co. Flow cytometry was purchased from BD Biosciences. Mini-Protean Tetra electrophoresis and blotting system were purchased from Beijing LIUYI Biotechnology Co. ChemiDoc™ XRS+ imaging system was purchased from Bio-Rad Co.

Western blot

Western blot was used to detect the protein expressions of HDAC1 in hepatoma cells after transfection. Briefly, hepatoma cells were seeded in 96-well plates at 100 μL per well. When cell confluence reaches 70%, protein in the whole cell lysates were extracted using protein extraction kit (Beyotime). Protein concentration was determined by Bicinchoninic Acid (BCA) protein assay kit according to the manufacturer's instructions.

Equal amounts of the protein were separated by 10% sodium dodecylsulfate-polyacryl- amide gel (SDS-PAGE), transferred to polyvinylidene fluoride (PVDF) membranes, and probed with antibodies against CyclinD1, p21, p27, ZO-1 and Vimentin (dilution 1:1000) overnight at 4°C, followed by covering with 5% non-fat milk for blocking with gentle shaking for 1 hour. Wash three times with TBST for 5 min each time. After that, appropriate horseradish peroxidases (HRP)-linked secondary antibodies (dilution 1:2000) were incubated for 2 h at room temperature. The immuno-reactive pro-

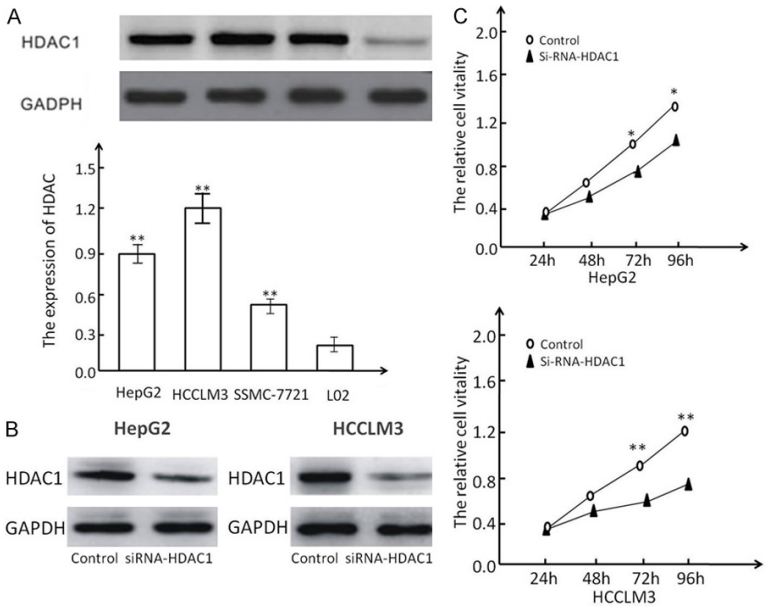

Figure 1. Silencing effects of HDAC1-siRNA and its effects on cell viability. A. Protein expressions of HDAC1 in hepatoma cells and esophageal cells were determined by Western Blotting. ** represents $p < 0.01$; * represents $p < 0.05$; B. Protein expressions of HDAC1 in hepatoma cells were determined by Western Blotting; C. Effects of siRNA-HDAC1 on cell viability of hepatoma cells and effects of siRNA-HDAC1 on HDAC1 expression of hepatoma cells. ** represents $p < 0.01$; * represents $p < 0.05$.

teins were detected with Pierce ECL Western blotting substrate (1:1, Reagent A:B) and visualized by ChemiDoc™ XRS + imaging system.

MTT assay

The hepatoma cells were seeded in 96-well plates. When the cell confluency reached 70%, cells were transfected with scrambled siRNA or HDAC1 siRNA at 50 and 100 nmol for 24 h, 48 h, 72 h and 96 h by using Lipofectamine 2000, and then 20 μL MTT solution (5 mg/ml) was added to each well and incubated for another 4 h at 37°C. After incubation, medium was removed and DMSO was added to dissolve purple precipitates. Then plates were read at 570 nm using a Microplate Reader.

Wound healing assay

The hepatoma cells were seeded in chamber slides and cultured until they had reached confluency. Wounding was performed using a 10 ul micropipette tip. The medium was removed and the chamber slides were washed to remove the cells fragment. Then serum-free medium was added and the chamber slides were placed into a cell incubator. Serial images were taken at 0h, 12 h and 24 h.

Transwell assay

When the cell confluency reached 70%, transfection of cells with scrambled siRNA or HDAC1 siRNA at 50 and 100 nmol for 72 h. For the migration assay, the cells were harvest and added in the top chamber and DMEM with 0.5% FBS were added in the bottom chamber, incubating for 24 h to allow migration. After washing, the cells were fixed with 10% paraformaldehyde and stained with 0.05% crystal violet. The total numbers of cells that have invaded were calculated under microscope. The average numbers of cells that have invaded were calculated basing on five random visual fields.

For the cell invasion assay, extracellular matrix (B&D Co.) was evenly spread on the invasion chambers, forming gel for further use. Calculate the average number of cells per field of view, which represents invasion ability of the cells.

Cell cycle analysis and detection expression of EMT-related protein

The hepatoma cells were seeded in 96-well plates. Each well contains 100 μL and each group has four plates. When the cell confluency reached 70%, cells were transfected with scrambled siRNA or HDAC1 siRNA at 50 and 100 nmol. After 72 h, cells were harvested and centrifuged. RIPA lysis solutions were added and every 10 mins placed on the vortex for 30 s. This process lasts for 40 mins. The supernatant containing whole cell lysate was extracted after centrifugation at 1000 rpm at 4 degree. Protein concentration was determined by BCA protein assay kit (Beyotime). After the protein was transferred to the PVDF membranes, primary antibody (1:100) was added and incubated overnight at 4 degree. After washing, secondary antibody (1:100) was added and incubated at 1 hour at room temperature. After

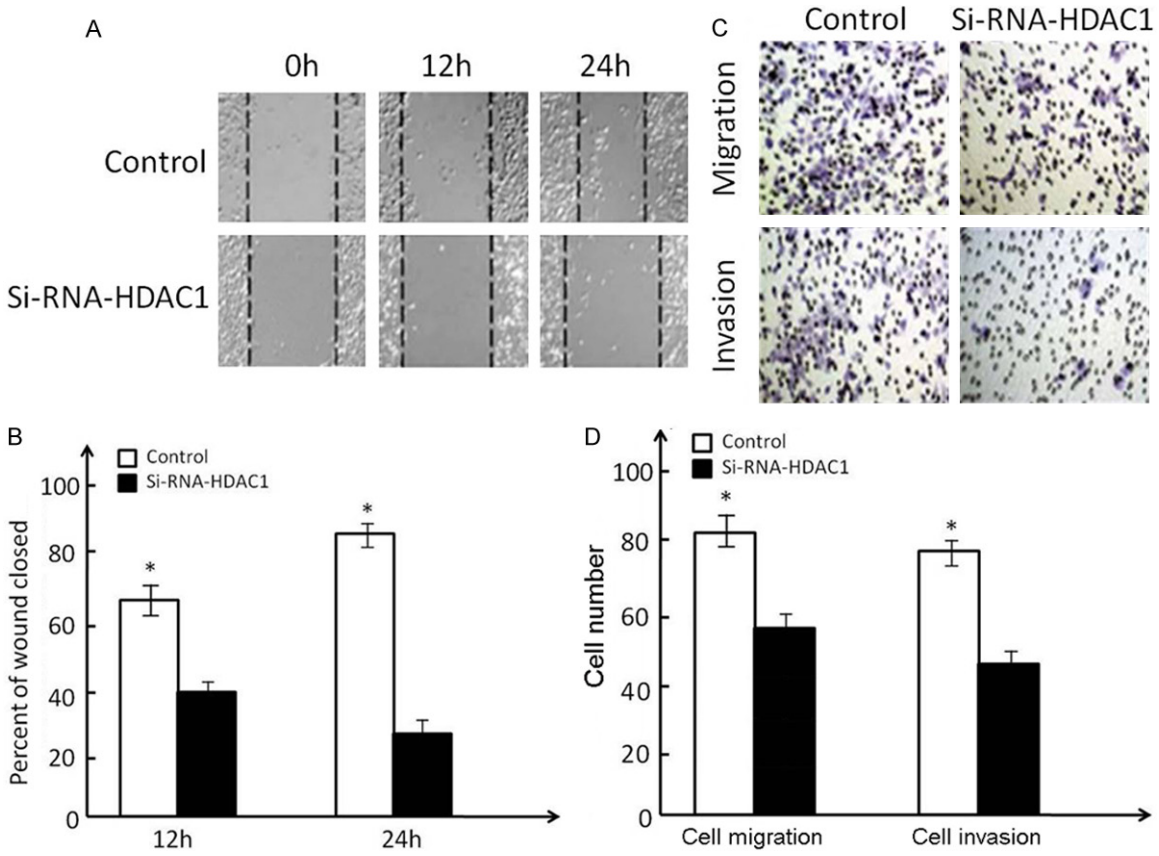

Figure 2. Effects of siRNA-HDAC1 on cell migration and invasion. A. Effects of siRNA-HDAC1 on migration of hepatoma cells. After 24 h and 48 h, there is statistical difference between siRNA-HDAC1 treated group and control group. B. There is statistical difference of migrated cell numbers between two groups. $P < 0.05$. C. Effects of siRNA-HDAC1 on invasion of hepatoma cells. D. There is statistical difference between two groups. $P < 0.01$.

washing, the immuno-reactive proteins were visualized by ChemiDoc™ XRS + imaging system.

Immunofluorescence assay

Cells were incubated in 100% methanol (chilled at -20°C) at room temperature for 5 min. After that, cells were fixed with 4% paraformaldehyde for 10 min at room temperature. Antigen retrieval was performed by heating samples for 5 min. Then the cells were incubated with 1% BSA for 30 min to block unspecific binding of the antibodies. After the temperature is cool down, cells were washed 3 times with PBS for 5 min. Blocking the samples with 10% BSA for 50 min and then stained with DAPI (1:100).

Apoptosis detection

The cells were collected by centrifugation at 800 rpm for 10 min. After that, the cells were washed and fixed at -20 degree overnight. The cells were resuspended with 100 μL Annexin-V-Fluos containing 10 μL FITC-labeled Annexin-V and 10 μL PI. After 7 min of incubation in dark, the cells were examined by flow cytometry.

Statistical analysis

Statistical analyses were performed using SPSS 11.0 (SPSS, Chicago, IL, USA). Statistical analysis was performed using one-way analysis of variance for multiple comparisons, followed by Student-Newman-Keuls test to evaluate the significance of differences between two groups. A *p*-value less than 0.05 was considered statistically significant.

Results

HDAC1 is overexpressed in hepatoma cells and siRNA-HDAC1 inhibits cell viability of hepatoma cells

The expressions of HDAC1 in hepatoma cells including HepG2, HCCLM3 and SSMC-7721 were significantly increased in comparison to LO2 cell lines (As shown in **Figure 1A**). Additionally, the expressions of HDAC1 in

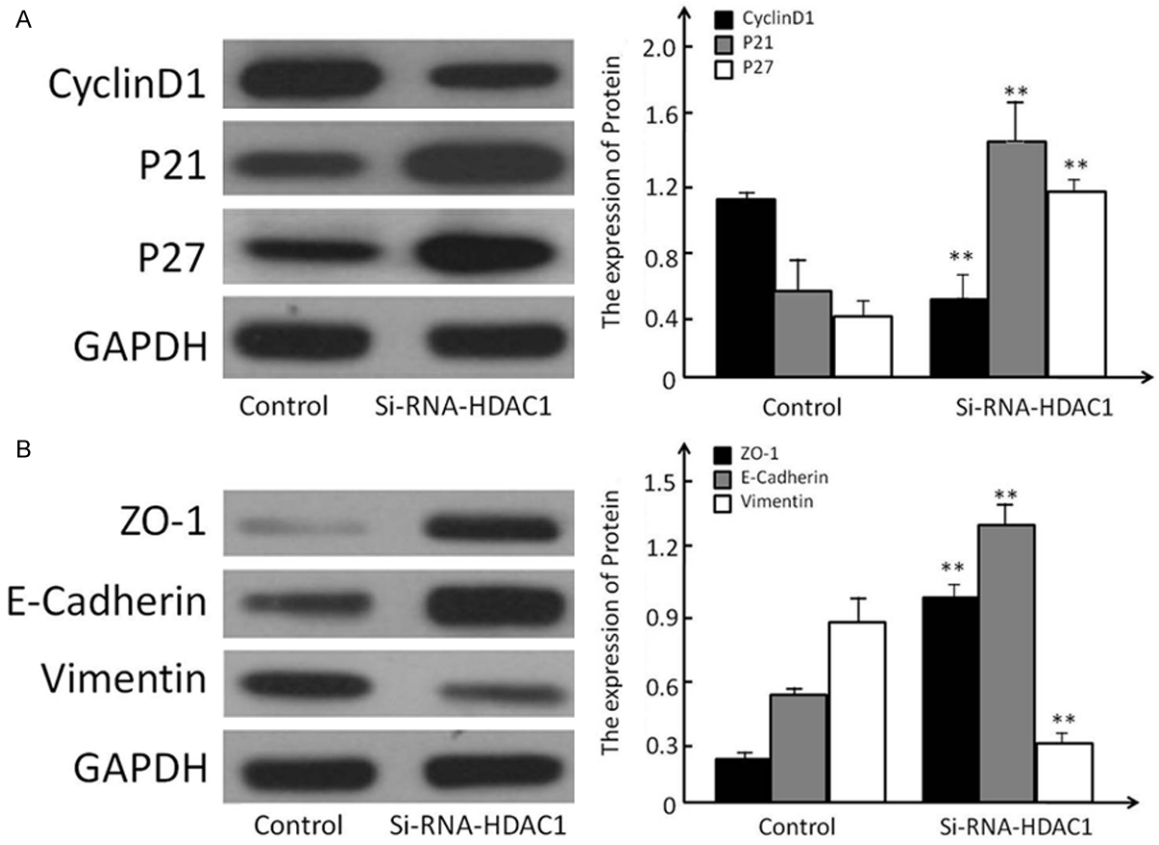

Figure 3. Effects of si-RNA-HDAC1 on expression of cell cycle related proteins and EMT related proteins. A. Effects of si-RNA-HDAC1 on expression of cell cycle related proteins in siRNA-HDAC1 treated group and control group, $p < 0.01$. B. Effects of si-RNA-HDAC1 on expression of EMT related proteins siRNA-HDAC1 treated group and control group, $p < 0.01$.

HepG2 and HCCLM3 cells are the highest. The western blotting showed that siRNA-HDAC1 treatment significantly decreased expressions of HDAC1 in HepG2 and HCCLM3 cells (As shown in **Figure 1B**). The MTT assay further confirmed that the cell viability of HCCLM3 cells were decreased than that of HepG2 cells after transfection 72 h and 96 h (As shown in **Figure 2C**), indicating that siRNA-HDAC1 treatment has best silencing and inhibition effects on HCCLM3 cells. Therefore, HCCLM3 cells were chosen for further study.

siRNA-HDAC1 inhibits cells migration and invasion of hepatoma cells

As shown in **Figure 2A**, wound healing assay showed that treatment with siRNA-HDAC1 significantly decreased migrated cell numbers in comparison to control group ($p < 0.01$). As shown in **Figure 2B**, transwell assay proved that treatment with siRNA-HDAC1 significantly decreased migrated cell numbers in comparison to control group ($p < 0.01$). These results indicated that siRNA-HDAC1 decreased expression of HDAC1, further attenuating cells migration and invasion.

siRNA-HDAC1 blocks cell cycle and EMT progression of hepatoma cells

As shown in **Figure 3**, when compared with control group, expression of cyclin D1 was significantly decreased ($p < 0.01$), and expressions of p21 and p27 were significantly increased ($p < 0.01$) in the siRNA-HDAC1 treated group. In addition, expressions of ZO-1 and E-cadherin were significantly decreased ($p < 0.01$) and expressions of Vimentin were significantly decreased ($p < 0.01$) in HCCLM3 cells.

siRNA-HDAC1 promotes cell apoptosis of hepatoma cells

Immunofluorescence assay showed condensation of cell nucleus which indicates cell apoptosis in siRNA-HDAC1 treated group. However, as shown in **Figure 4A**, the morphology of cell

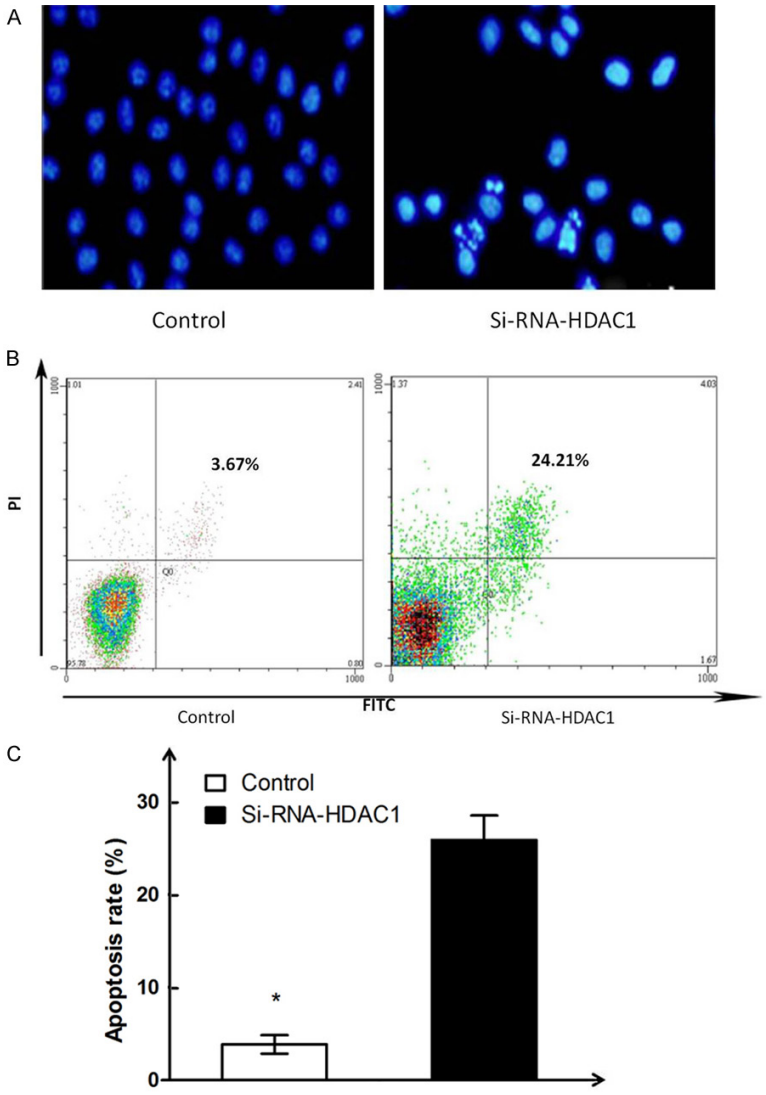

Figure 4. Effects of siRNA-HDAC1 on apoptosis of HCCLM 3 cells. A. The numbers of apoptotic cells increased in siRNA-HDAC1. Morphology of cell nucleus was observed by using immunofluorescence assay. B. Cell apoptosis was detected by flow cytometry. C. Quantification of cell apoptosis. The numbers of apoptotic cells in siRNA-HDAC1 treated group significantly higher than that of in control group.

nucleus was normal in control group. As shown in **Figure 4B** and **4C**, the numbers of apoptotic cells in siRNA-HDAC1 treated group were significantly increased than that of in control group.

Discussion

Many studies demonstrated that dysfunction of HDAC1 is associated with the occurrence and development of a series of cancers [9]. Quint et al., found that HDAC1 is expressed in primary esophageal tumor site and nearby normal tissues among 170 surgical specimens [10]. The expression level of HDAC1 is significantly higher in tumor site, reflecting malignance of tumor. Cheng et al., confirmed that expressions of HDAC1 in fiber cells in the normal esophageal tissues are higher than those in fiber cells in the esophageal cancer tissues [11]. Additionally, several researches demonstrated that HDACs inhibitors can suppress cell growth and differentiation, leading to cell apoptosis in malignant tumor including esophageal, lung, cervical, prostate and breast cancer [12]. These results confirmed the relationship between HDAC1 overexpression and development of many cancers. However, the relationship between HDAC1 overexpression and hepatoma is still unclear. Therefore, in the present study, the HDAC1 expression levels in three liver cancer cell lines were detected by using RT-PCR and western blot. The results showed significant increases of HDAC1 expression in hepatoma cells including HepG2, HCCLM3 and SSMC-7721 cells. Then hepatoma cells were transfected with siRNA-HDAC1 by using Lipofectamine 2000. Finally, HCCLM3 cell line was chosen for the following study based on results acquired by using western blot and MTT assay.

Lei et al., found HDAC1 is overexpressed in invasive hepatoma tissues. Besides, they found that cell migration was suppressed by silencing HDAC1 by using siRNA interfere. Additionally, another study found that the HDAC1 inhibitor reverses the process of EMT in prostate cancer, indicating that HDAC1 may affect the growth and metastasis of hepatoma [13-15]. Many evidences demonstrated that EMT plays an important role in the growth, metastasis and invasion of tumors [16]. Cyclin D1 is a key protein required for progression through the G1 phase of the cell cycle. During the G1 phase, it

is synthesized rapidly and accumulates in the nucleus, and is degraded as the cell enters the S phase [17]. Cyclin D1 dimerizes with CDK4/6 to regulate the G1/S phase transition and entry into the S-phase. The present study showed that HDAC1siRNA transfection down-regulated the expression of CyclinD1, suggesting that HDAC1 affects cell proliferation by regulation of cell cycle.

Peripheral membrane protein ZO-1 is the platform for tight junction. The structural and functional change of ZO-1 leads to a loss of tight junction [18]. The abnormal expression of E-cadherin in the tissues was proved to induce tumorigenesis. Many study demonstrated that abnormal expression of E-cadherin may contribute to the occurrence and metastasis of tumor in hepatoma [19]. In mesenchymal tumors, overexpression of vimentin promotes tumor invasion and metastasis, further reverses expression of EMT related proteins and remarkable suppresses tumor invasion and metastasis. Furthermore, many studies demonstrated that HDAC1 is closely associated with EMT. Von et al., found that HDAC1 is crucial in pancreatic cancer metastasis and EMT due to absence of snail and E-cadherin [20]. The present study found the expression of Vimentin was decreased and expressions of Z0-1 and E-cadherin were decreased in siRNA-transfected HCCLM3 cells. Immunofluorescence assay showed that condensation of cell nucleus in siRNA-HDAC1 treated group. Flow cytometry assay demonstrated that siRNA-HDAC1 treated group has higher numbers of apoptotic cells than that of in control group.

In conclusion, we found that HDAC1 is overexpressed in hepatoma cell lines. HDAC1 siRNA significantly down regulated expression of HDAC1, further inhibiting the proliferation, metastasis and invasion of HCCLM3 cells. Transfection of siRNA-HDAC1 decreased expressions of CyclinD1 and increased the expressions of p21 and p27. In addition, transfection of siRNA-HDAC1 down-regulated expression of vimentin and up-regulated expression of EMT related proteins including ZO-1 and E-cadherin. These results demonstrated that transfection of HDAC1 siRNA affect proliferation of hepatoma cells though regulation of cell cycle and modulation of expression of EMT-related protein. The results shown in present study provide potential evidences for molecular targeting therapy of hepatoma.

Disclosure of conflict of interest

None.

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