Original Article
A novel treatment strategy in hepatocellular carcinoma by down-regulation of histone deacetylase 1 expression using a shRNA lentiviral system

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Abstract: Up to now, effective treatment methods for hepatocellular carcinoma (HCC), the fifth most prevalent cancer worldwide, have remained very limited. Previous studies have shown that histone deacetylase 1 (HDAC1) is highly expressed in HCC. The adoption of HDAC inhibitors in HCC treatment has also been studied, however, only moderate efficacy was observed. In the current study, using a clinically approved shRNA lentiviral system, we investigated whether the down-regulation of HDAC1 on mRNA level could suppress HCC progression. Our results showed that HDAC1 shRNA lentivirus infection could significantly reduce HCC Hep3B cell viability but have little impact on normal liver cell THLE-3. Cell cycle analysis revealed that HDAC1 shRNA lentivirus treatment could arrest HCC cells at G1 phase, which was probably achieved by modulating expression of cell cycle-related proteins including Cyclin D1, p21 and p27. Invasion assay showed that HDAC1 knock-down could dramatically reduce the invasiveness of HCC cells, which was correlated to the altered expression of some epithelial-mesenchymal transition related proteins including ZO-1, E-cadherin and Vimentin. Taken together, our findings have shown that down-regulation of HDAC1 on mRNA level using shRNA lentiviral system might be a novel alternative treatment strategy for HCC.

Keywords: Hepatocellular carcinoma, histone deacetylase 1, shRNA lentiviral system

Introduction
Hepatocellular carcinoma (HCC), the fifth most prevalent cancer worldwide [1], is a fatal malignance with fast disease progression [2]. Since the time of diagnosis, the life expectancy of this disease is about 6 months [3]. With the development of technology, the treatment options for HCC has been increasing [4, 5], however, the curative treatments for HCC are still only resection and transplantation [6]. Unfortunately, the reoccurrence rate of HCC patients with resection and transplantation is considerably high, rendering the mortality rate of this disease almost equals to the incidence rate [6, 7]. Consequently, novel HCC treatment strategies with high efficacy are essentially needed to be developed.

Human carcinogenesis is usually triggered by some aberrant biological events, among which, epigenetic abnormality is one of great importance [8]. Unlike genetic modification, epigenetic alteration could result in aberrant expression of various genes without directly introducing changes in DNA sequence [9]. Histone acetylation, one of the recently discovered epigenetic modifications involving in HCC progression, is regulated by histone acetylases (HATs) and histone deacetylases (HDACs). Previous studies have shown that HDACs involve in the pathogenesis of many cancers [10-12] and the adoption of HDAC inhibitors as cancer treatment remedies have been investigated in preclinical and clinical studies [13]. As one of the most important members in the HDAC family, HDAC1 has been reported to be highly expressed in HCC cell lines and primary tissues [14]. Moreover, study by Quint K and colleagues has shown that high HDAC1 is associated with low tissue differentiation and low survival rate [15]. Recent studies have also investigated the efficacies of some HDAC inhibitors in HCC, however, only moderate efficacy was observed [16-19].

In the current study, we tested the efficacy of HDAC1 down-regulation on mRNA level in HCC
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using a shRNA lentiviral system. Introduction of shRNA into cells using lentiviral vectors have been approved to be an efficient and safe technology in both pre-clinical and clinical trials [20]. Herein, we specifically down-regulated HDAC1 on mRNA level with a shRNA lentiviral system and investigated the effect of down-regulation in HCC cell viability, invasion and survival. The results of our study implied a novel treatment strategy with great potential in HCC.

Materials and methods

Cell lines, shRNA lentiviral vectors

HCC cell lines HepG2, Hep3B, Huh7, PLC/PRF/5, SK-Hep-1 and human normal liver cell line THLE-3 were purchased from Cell Bank of Chinese Academy of Sciences and cultured in DMEM supplemented with 10% Fetal bovine serum (GIBICO, Invitrogen) and penicillin (100 U/ml, Sigma-Aldrich), and streptomycin (100 mg/ml, Sigma-Aldrich). Human HDAC1 shRNA Lentiviral Particles and Control shRNA Lentiviral Particles were purchased from Santa Cruz Biotechnology, Inc.

HDAC1 knock-down using shRNA lentiviral system

Cells were pre-seeded one day before shRNA treatment in 6-well plates (1×10^5 cells/well) and then incubated with various doses of HDAC1 shRNA lentiviral particles or control shRNA lentiviral particles for up to 96 h. The HDAC1 knock-down efficiency was determined by RT-PCR and Western blot.

RT-PCR

Total RNA was first extracted using Trizol agent (Invitrogen) and then reverse-transcription and target gene amplification were performed using a one-step RT-PCR kit (Qiagen). After amplification, PCR products were separated by gel electrophoresis and semi-quantification of gene expression was conducted using Image J. The following primers were used in RT-PCR assay: HDAC1: forward 5'-AACTGGGGACCTACGG-3' and reverse 5'-ACTTGGCGTGTCCTT-3'; Internal control GAPDH: forward: 5'-AGCCACATCGCTCAGACA-3' and reverse 5'-TGGACTCCACGAGGAAGACT-3'.

Western blot

Cells with or without shRNA lentiviral particles treatment were first lysed and separated by 12% SDS-PAGE and then transferred onto a PVDF membrane. Non-specific binding sites of the membrane was blocked with 5% non-fat milk. Post blocking, membrane was then incubated with primary antibodies and corresponding secondary antibodies for 2 h and 1 h at room temperature, respectively. After incubation, membrane was extensively washed with PBS-T and the immune-bands was visualized using ECL chemiluminescence kit (Pierce, Invitrogen) under a CCD camera (Bio-Rad). The following primary antibodies were used: rabbit anti-Cyclin D1, anti-p21, anti-p27 and anti-ZO-1 were from Epitomics while rabbit anti-E-cadherin, anti-Vimentin and anti-GAPDH were from Abcam.

Cell viability assay

Cell viability was determined with a MTT assay kit (Invitrogen) according to the manufacturer's instructions. In brief, cells with or without shRNA lentiviral particle treatment were washed with PBS and then incubated with MTT solution for 4 h. After incubation, medium was removed and DMSO was added and incubated for 10 min at 37°C. Finally, samples were mixed and OD540 nm value was read.

Cell cycle assay

Cells with or without shRNA lentiviral particles treatment were first harvested and fixed in 70% ethanol, and then stained with FxCycle™ PI/RNase Solution (Invitrogen) for 30 min in the dark. After staining, cells were analyzed on a BD FACS Aria III platform.

Cell invasion assay

Cell invasion assay was conducted using Matrigel as previously described with modifications [21]. In brief, the Matrigel Transwell system was established by coating 1 mg/ml Matrigel (Corning) onto the 8 mm pore-size Transwell inserts (Corning Costar). For invasion assay, the Matrigel coated upper chambers were added with cells with or without shRNA lentiviral particle treatment while the lower chambers were filled with complete medium. The invasion system was then incubated in a
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37°C 5% CO₂ incubator for 24 h. After incubation, non-invading cells on the Matrigel coating were removed and the Matrigel was fixed with paraformaldehyde and stained with crystal violet. The invaded/migrated cells were then visualized under microscope.

Statistical analysis

Data were presented as mean ± SD. Student’s t test was used in this study to compare the difference between two groups and a p value < 0.05 was considered statistically significant. All statistical analysis was performed with SPSS 17.0 (SPSS, Inc.).

Results

HDAC1 expression was increased in HCC cell lines

We first examined the expression difference between HCC and normal liver cell lines. Five HCC cell lines (HepG2, Hep3B, Huh7, PLC/PRF/5, SK-Hep-1) and one human normal liver cell line THLE-3 were tested. As shown in Figure 1A and 1B, the mRNA levels of HDAC1 were significantly increased in all HCC cell lines then normal liver cell line THLE-3. Notably, although all HCC cell lines, comparing to THLE-3, showed increased HDAC1 expression on mRNA level, there were still slight expression differences among these five HCC cell lines, with Hep3B exhibiting the highest HDAC1 expression. HDAC1 expression on protein level was also determined and similar results were observed (Figure 1C and 1D).

The effect of HDAC1 shRNA lentiviral particle treatment on HCC cell viability

Since HDAC1 expression in HCC cells was increased, we hypothesized that the reverse of the abnormal expression alteration might have inhibitory effect on HCC cell events, including survival, proliferation and invasion. Therefore, we down-regulated HDAC1 expression on mRNA level with a shRNA lentiviral system and observed the changes in HCC cells.

First, we tested the effect of lentivirus dose and infection time on cell viability. As shown in Figure 2A, at fixed infection time (72 h), the viability of Hep3B cells decreased when the dose of HDAC1 shRNA lentivirus increased. However, Hep3B cells infected with control shRNA lentivirus and THLE-3 cells infected with HDAC1 shRNA lentivirus demonstrated little
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Figure 2. The effect of HDAC1 shRNA lentivirus infection dose and time on Hep3B cell viability. A. Cell viability after shRNA lentivirus infection at increasing doses (Left) and time (Right). *P < 0.05; **P < 0.01. B. HDAC1 expression on mRNA (Left) and protein (Right) levels after shRNA lentivirus infection at 5 MOI for 72 h. Representative result is shown.

Table 1. HDAC1 down-regulation arrested HCC cells at G1 phase

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<th>G1 (%)</th>
<th>S (%)</th>
<th>G2 (%)</th>
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<tr>
<td>Control shRNA</td>
<td>53.48±7.63</td>
<td>33.92±4.02</td>
<td>12.60±5.07</td>
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<tr>
<td>HDAC1 shRNA</td>
<td>74.32±6.59**</td>
<td>23.11±2.03*</td>
<td>2.57±0.24*</td>
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*P < 0.05; **P < 0.01.

change in cell viability when virus dose was no more than 5 multiplicity of infection (MOI), and yet the cell viability decreased when virus dose continue to increase (Figure 2 left). At fixed infection dose (5 MOI), when infection time increased, the viability of Hep3B cells treated with HDAC1 shRNA lentivirus was decreased dramatically while that of Hep3B cells treated with control shRNA lentivirus or THLE-3 cells treated with HDAC1 shRNA lentivirus exhibited no apparent decrease when infection time increased up to 72 h (Figure 2 right). The results above indicated that when cells treated with shRNA lentivirus at an MOI of 5 and infection time of 72 h, HDAC1 knock-down in Hep3B cells had significant impact on cell viability while no apparent change was observed in either control shRNA treated Hep3B or HDAC1 shRNA treated THLE-3 cells. Therefore, the infection dose of 5 MOI and infection time of 72 h was adopted for the down-stream experiments. We further confirmed the HDAC1 expression in Hep3B cells on both mRNA and protein levels after shRNA lentivirus infection (5 MOI, 72 h) and our results showed that HDAC1 mRNA (Figure 2B left) and protein (Figure 2B right) were both significantly decreased. Taken together, the results herein suggested that specifically down-regulation of HDAC1 on mRNA level could affect the viability of HCC cells but not normal liver cells.

HDAC1 down-regulation arrested HCC cells at G1 phase

Since HDAC1 down-regulation reduced HCC cell viability, indicating cell proliferation might be affected, we subsequently investigated whether abnormality in cell cycle was generated in HCC cells by HDAC1 shRNA treatment. As shown in Table 1, in control shRNA group, cells at G1, S and G2 phase were 53.48%, 33.92% and 12.60%, respectively. Whereas in HDAC1 shRNA group, most of the cells were arrested at G1 phase (74.32%).

We further tested whether some cell G1 phase-related proteins were involved in the HDAC1
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Three key proteins were studied in the current study: Cyclin D1, p21 and p27. Cyclin D1 is a protein required for cell progression through G1 phase into S phase [22], p21 can mediate p53-dependent cell cycle G1 phase arrest [23], while over-expressed p27 can also cause cells to arrest in G1 phase [24]. Western blot analysis revealed that the expression of Cyclin D1 was decreased while that of p21 and p27 was increased after HDAC1 knock-down, indicating that HDAC1 down-regulation might indirectly altered the expression of cell cycle proteins including Cyclin D1, p21 and p27, rendering cells at G1 phase arrest (Figure 3).

HDAC1 down-regulation decreased HCC cell invasiveness

Cancer cell invasion is an important indicator of cancer progression, thus we further investigated whether HDAC1 knock-down has suppressive effect on HCC cell invasiveness. As shown in Figure 4A and 4B, after HDAC1 shRNA lenti-

Figure 3. The effect of HDAC1 down-regulation on the expression of cell cycle related proteins. A. Representative result is shown. B. Gray scale analysis. Mean ± SD of three independent experiments is shown. **P < 0.01.

Figure 4. HDAC1 down-regulation suppressed Hep3B cell invasion. A. Representative result is shown. B. Mean ± SD of cell numbers in five random high-fields from three independent experiments is shown. ***P < 0.001.
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virus treatment, the invasiveness of Hep3B cells were dramatically reduced, comparing to that of control shRNA treated cells.

Further study was also conducted to explore the possible mechanism underlying HDAC1 knock-down mediated suppression on HCC cell invasion. Epithelial-Mesenchymal Transition (EMT) has been shown to be involved in the initiation of metastasis for cancer progression [25]. Consequently, we investigated whether HDAC1 knock-down induced expression alteration of EMT-related proteins. Tight junction protein ZO-1 is involved in the tight junction between cell membranes [26], E-cadherin, a type I transmembrane protein, plays important roles in cell adhesion [27], while Vimentin has been shown to promote tumor invasion and metastasis [28]. Our results demonstrated that, after HDAC1 shRNA lentivirus treatment, the expression of ZO-1 and E-cadherin increase while that of Vimentin decreased (Figure 5A and 5B), indicating that HDAC1 down-regulation might suppress HCC cell invasion through alteration on the expression of EMT-related proteins.

Taken together, our study herein demonstrated that down-regulation of HDAC1 on mRNA level using shRNA lentiviral system could reduce cell viability of HCC cells but not normal liver cells. Moreover, HDAC1 down-regulation could arrest HCC cells at G1 phase probably through regulating cell cycle-related protein including Cyclin D1, p21 and p27, and could suppress the invasiveness of HCC cells via modulation on the expression of EMT-related proteins. The findings of the current study have shown that down-regulation of HDAC1 on mRNA level using shRNA lentiviral system might be a novel alternative treatment strategy for HCC.

Discussion

HDAC abnormality has been described in many types of cancers and for some cancers, HDAC inhibitors exhibit very promising results. In HCC, the most studied HDAC family member is HDAC1, which expressed in aberrantly high level in HCC cells. The use of HDAC inhibitors has also been investigated in treating HCC, however, the efficacy has shown to be only moderate. shRNA lentiviral system is a clinically approved method to introduce specific gene knock-down and has been widely used in various kinds of research. In the current study, we, for the first time, have tested the effect of HDAC1 down-regulation on mRNA level using a shRNA lentiviral system in suppressing HCC progression. Our results have shown that HDAC1 shRNA lentiviral treatment could specifically reduce HCC cell viability and invasiveness but have no apparent impact on normal liver cells. The findings of the current study have shown that down-regulation of HDAC1 on mRNA level using shRNA lentiviral system might be a novel alternative treatment strategy for HCC.

HDAC is a big family and has many family members, which can be grouped into four classes. Knock-down of HDACs in tumor cells has been previously studied, however, only knock-down of specific HDAC member could inhibit tumor cell proliferation [29]. HDAC1 is the most extensively studied member in HCC and our results
have shown that down-regulation of this protein on mRNA level could significantly inhibit HCC cell viability and invasion. Aside from HDAC1, other HDAC members, including HDAC3 and HDAC5, have also been described to be involved in HCC in previous studies [30, 31]. Whether knock-down of other HDACs or the combination of a few HDACs could also demonstrate inhibitory effect in HCC progression remains to be further determined.

It has been previously reported that HDAC1 deficiency in tumor cells could arrest cells at G1 or G2/M phase and consequently inhibit cell proliferation [32]. In the current study, we also observed cell G1 phase arrest in HDAC1 shRNA lentivirus treated HCC cells. We further investigated potential mechanisms by determining the expression profile change of G1 phase related key proteins. We found out that HDAC1 knock-down inhibited the expression of Cyclin D1 while promoted the expression p21 and p27. Cyclin D1, a key protein in G1 phase, is synthesized in G1 phase and essential for cell through G1 into S phase, while both p21 and p27 are negative regulators in cell cycles, which could arrest cells at G1 phase. Although more detailed pathway research needed to be further conducted, we can conclude that HDAC1 knock-down arrests cell at G1 phase and it is related to the expression alteration of Cyclin D1, p21 and p27.

Cancer cell invasion is a complex process and is mediated by the aberrant expression of a series of proteins. EMT proteins are closely related to the initiation of tumor metastasis, and therefore, they are usually adopted as biomarkers for tumor invasion and transfer. ZO-1, E-cadherin and Vimentin are three important EMT proteins. Previous study has described that loss of E-cadherin is correlated with tumor invasion, metastasis and bad prognosis in HCC [33]. While in pancreatic carcinoma, HDAC1 and Snail are involved in the decreased expression of E-cadherin [34]. Moreover, in TGF-β-induced liver EMT mouse model, HDAC1 can inhibit the promoter activity of ZO-1 and E-cadherin [35]. Our current study has revealed that down-regulation of HDAC1 on mRNA level could significantly reduce HCC cell invasion and also that HDAC1 shRNA lentiviral treatment increased the expression of ZO-1 and E-cadherin but decreased that of Vimentin. Therefore, it is reasonable to conclude that HDAC1 down-regulation might suppress HCC cell invasion through alteration on the expression of EMT-related proteins and further research underlying detailed mechanisms is also warranted.

Taken together, our study herein demonstrated that down-regulation of HDAC1 on mRNA level using shRNA lentiviral system could reduce cell viability of HCC cells but not normal liver cells. Moreover, HDAC1 down-regulation could arrest HCC cells at G1 phase and could suppress the invasiveness of HCC cells. The findings of the current study have shown that down-regulation of HDAC1 on mRNA level using shRNA lentiviral system might be a novel alternative treatment strategy for HCC.

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Disclosure of conflict of interest

None.

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References

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