Int J Clin Exp Med 2016;9(11):21482-21489
www.ijcem.com /ISSN:1940-5901/IJCEM0033213

Original Article

Analysis of miRNAs related to abnormal HDAC1 expression in hepatocellular carcinoma

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Received June 3, 2016; Accepted August 8, 2016; Epub November 15, 2016; Published November 30, 2016

Abstract: Histone de-acetylation is closely related to the occurrence and development of cancers. The abnormal expression of HDACs may cause the histone acetylation imbalance. In this study, the protein expression of HDAC1 and HDAC2 was detected in the hepatocellular carcinoma (HCC) tissues of 60 cases. Our results showed the expression of HDAC1 and HDAC2 increased significantly in HCC as compared to adjacent normal tissues. Thus, we speculate that both HDAC1 and HDAC2 are associated with the progression of HCC. Then, qRT-PCR was performed to detect the expression of miR-29b, miR-34a, miR-449a and miR-520h in these HCC tissues, and results showed all these microRNAs showed markedly decreased expression in HCC as compared to adjacent normal tissues ($P<0.05$). In HCC, HDAC1 expression was negatively associated with the expression of 4 microRNAs. Thus, the abnormal HDAC1 expression in HCC might be regulated by microRNA. Dual luciferase experiment indicated that miR-34a and miR-449a were the microRNAs acting on the 3'UTR of HDAC1. Our results suggest that microRNA is able to regulate HDAC1 expression, exerting anti-tumor effects, which provides a new clue for the diagnosis and treatment of HCC.

Keywords: Hepatocellular carcinoma, microRNA, HDAC1, HDAC2

Introduction

Hepatocellular carcinoma (HCC) accounts for about 90% of primary malignancies in the liver and has high morbidity and high mortality [1]. HCC has been the 6th most common malignancy world wide [2]. HCC has the characteristics of insidious onset, rapid progression, high malignancy, high post-operative recurrence and metastasis rates and poor sensitivity to radiotherapy and chemotherapy. Thus, the therapeutic efficacy is still poor for HCC patients and the 5-year survival rate is very low [3]. In addition, radical surgery is feasible in only 30-40% of HCC patients [4]. Thus, to identify specific markers is crucial for the early diagnosis and treatment of HCC.

microRNA (miRNA) is a group of small non-coding RNA molecules containing 18-25 nucleotides. In the eukaryotes, microRNAs may regulate its target genes to affect the proliferation, differentiation, apoptosis, infiltration and migration of cells [5]. Studies have reported that microRNAs can regulate some genes closely related to cancers, which may provide a way for the diagnosis and treatment of cancers [6]. Several studies have shown that miRNAs play important roles in the proliferation, differentiation, invasion and metastasis of HCC cells, and thus miRNAs have the potential for the early diagnosis and individualized therapy of HCC [7]. Hypo-acetylation and hypermethylation of the histone are the characteristics of cancer cells. The histone deacetylase (HDAC) is crucial in the process of protein acetylation, and abnormal HDACs expression may cause the imbalance of protein acetylation, which has been confirmed to be closely related to the occurrence and development of cancers. Histone deacetylase 1 (HDAC1) is one of HDACs found in the mammalians [8] and has abnormally high expression in the colon cancer, pancreatic cancer, lung cancer and liver cancer. To date, HDAC1 has been a focus in studies on cancers [9]. The HDAC1 expression is also regulated by miRNAs. It has been found that some miRNAs can regulate the HDAC1 expression to affect the biobehaviors of cancer cells. In the present study, the HDAC1 expression was detected in HCC, and miRNAs related to the regulation of

HDAC1 expression were also screened in HCC, which may provide new targets for the diagnosis and therapy of HCC.

Material and methods

Sample collection

HCC tissues were collected from 60 patients who received surgical intervention due to HCC in the Henan Provincial People's Hospital between June 2014 and June 2015, and informed consent was obtained before study. All the patients were diagnosed with HCC by ultrasound examination, CT and pathological examination on the basis of serum AFP level and medical history of hepatitis. The adjacent normal tissues (2 cm away from the cancer) free of cancer cells confirmed by pathological examination were also collected as controls. There were 51 males and 9 females with the median age of 51 years (range: 38-65 years). All the patients did not receive chemotherapy, radiotherapy and immune therapy before study. This study was approved by the Ethics Committee of Henan Provincial People's Hospital.

Cell line and materials

Human HCC cell line HepG2 cells were provided by the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were maintained in high glucose DMEM containing 10% fetal bovine serum (Gibco BRL, Gaithersburg, MD, USA) in a humidified environment with 5% CO_2 at 37°C.

Immunohistochemistry

HCC tissues were fixed in 4% formaldehyde, embedded in paraffin and cut into 3 μm sections. Immunohistochemistry was performed with EnVision K5007 kit (Dako) and antibodies against HDAC1 and HDAC2 (Abcam, Cambridge, UK). In negative control group, the primary antibody was replaced with PBS. Positive cells had brown or yellow-brown granules in the nucleus. Ten fields were randomly selected from each section, and a total of 100 cells were counted in each field. The proportion of positive cells was calculated: negative, <10%; positive, ≥10%.

RNA extraction and RT-qPCR

Target Scan (http://www.targetscan.org/), PicTar (http://pictar.org/) and miRanda http://www.microrna.org/microrna/) were employed for the prediction of target genes of HDAC1, and miRNAs complementary to the 3'UTR of HDAC1 were selected. After screening, miR-29b, miR-34a, miR-449a and miR-520h were found as targeted miRNA. Fluorescence quantitative PCR (RT-qPCR) was employed for the detection of miRNA expression in HCC tissues and adjacent normal tissues. Primers were provided by the Applied Biosystems Company (Foster City, CA, USA).

Total RNA was extracted with a kit according to the manufacturer's instructions (Qiagen, Venlo, Netherlands), and RNA concentration and purity were determined with the NanoDrop 1000 spectrophotometer. Then, RNA was reversely transcribed into cNDA with MMLV RTase cDNA Synthesis Kit according to the manufacturer's instructions (TaKaRa, Dalian, China). cDNA amplification was done with ABI Power SYBR-Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). A melt curve was delineated, RNU6B served as an internal reference, and the miRNAs expression was determined by comparisons of CT values with $2^{-\Delta CT}$ method.

Dual luciferase reporter assay

The binding sites of miR-34a and miR-449a to HDAC1 were analyzed, with miR-34a mimic and miR-449a mimic (Shanghai GenePharma Co. Ltd, Shanghai, China) as negative controls. Genome was collected from healthy subjects and the 3'UTR of HDAC1 was amplified by PCR. After retrieval and purification, it was connected to pmirGLO to construct pmirGLO-HDAC1-wt. The mutant primers targeting the seed region of HDAC1 3'UTR were designed and the mutant sequence of HDAC1 was amplified with over lap method and then connected to pmirGLO. The resultant vector was named pmirGLO-HDAC1-mut.

Dual luciferase reporter assay: HepG2 cells were seeded into 96-well plates. miR-34a mimics (or miR-449a mimic) and pmirGLO-HDAC1-wt were co-transfected into HepG2 cells in the presence of Lipofectamine™ 2000 (Invitrogen). miR-34a mimic (or miR-449a mimic) and pmirGLO-HDAC1-mut were co-transfected into HepG2 cells in the presence of Lipofectamine™ 2000 (Invitrogen). At 48 h, dual luciferase reporter assay was performed to detect the dual luciferase signals with a kit according to the manufacturer's instructions (Promega, Madison, WI, USA).

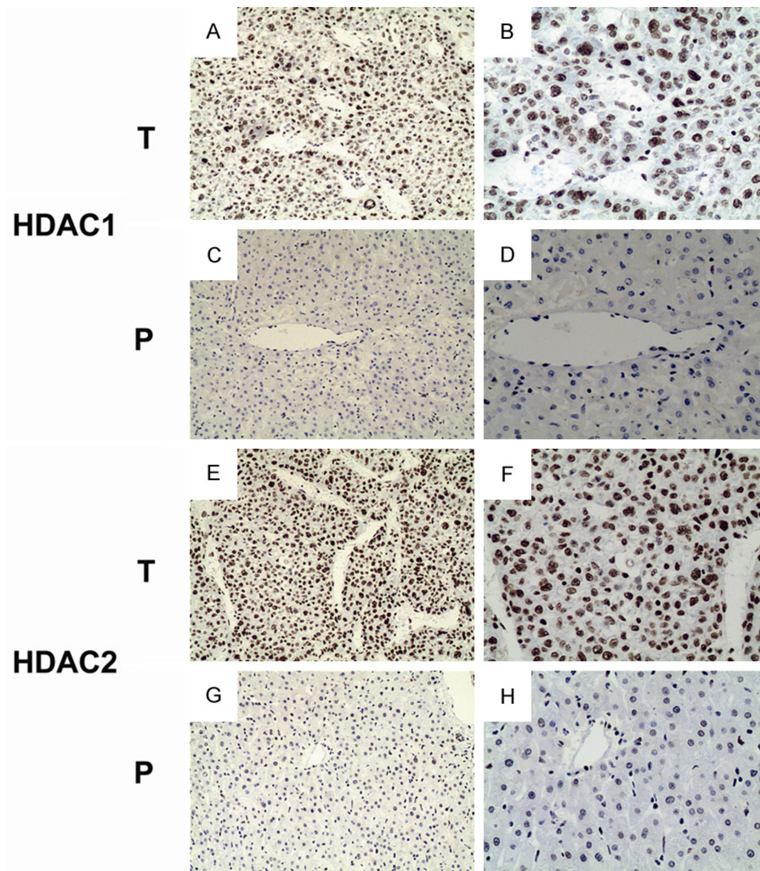

Figure 1. HDAC1 expression is mainly found in HCC tissues (A: ×100 and B: ×200 EnVision), and adjacent normal tissues are negative for HDAC1 (C: ×100 and D: ×200 EnVision). HDAC2 expression is mainly found in HCC tissues (E: ×100 and F: ×200 EnVision), and adjacent normal tissues are negative for HDAC2 (G: ×100 and H: ×200 EnVision).

Western blot

Total protein was extracted from tissues, subjected to SDS-PAGE and then transferred onto nitrocellulose membrane (Whatman, GE Healthcare, UK). The membrane was incubated with primary antibody (1:500; Abcam) in 5% non-fat milk at 4°C over night. After washing in TBST thrice (15 min for each), the membrane was incubated with horseradish peroxidase conjugated secondary antibody (IgG; 1:1000). Visualization was done with the chemiluminescence detection kit (Amersham Pharmacia Biotech, Piscataway, NJ). β-actin (Santa Cruz Biotechnology, SantaCruz, CA) served as an internal reference, and the relative protein expression of HDAC1 was determined.

Statistical analysis

Statistical analysis was performed with SPSS version 18.0. One way analysis of variance or chi square test were employed for the comparisons among groups. Quantitative data are expressed as mean ± standard deviation (mean ± SD). A value of $P<0.05$ was considered statistically significant.

Results

Expression of HDAC1 and HDAC2

Immunohistochemistry was performed to detect the protein expression of HDAC1 and HDAC2 in HCC tissues and adjacent normal tissues. HDAC1 and HDAC2 expression was mainly localized in the nucleus, and positive cells had dark brown or yellow brown granules in the nucleus. Of 60 tissues, 47 were positive for HDAC1 with the positive rate of 78.33%; of adjacent normal tissues, the positive rate was only 26.67% (16/60). Of 60 tissues, 49 were positive for HDAC2 with the positive rate of 81.67%; of adjacent normal tissues, the positive rate was only 28.33% (17/60). This suggested that HDCA1 and HDAC2 expression in HCC was significantly higher than in adjacent normal tissues ($P<0.05$), and the HDCA1 and HDAC2 protein expression was upregulated in HCC (**Figure 1**).

The correlation between clinicopathological characteristics and HDAC1/HDAC2 expression was further evaluated in these patients. Results showed the HDAC1 and HDAC2 expression was not associated with age, gender, lymph node metastasis and clinical stage of HCC (P<0.05). HDAC1 protein expression in well to moderately differentiated HCC was significantly lower than in poorly differentiated HCC (**Table 1**, $P<0.05$), but HDAC2 protein expression was not related to the differentiation degree of HCC (**Table 2**; P>0.05).

Expression of miRNA related to HDAC1

On the basis of results from prediction of targeted miRNAs, the expression of 4 miRNAs

Table 1. Correlation of clinicopathological characteristics with HDAC1 expression in HCC patients

Clinicopathological Characteristics	n	HDAC1 (+)	HDAC1 (-)	*P* value
Age (yr)				
≤50	22	17	5	0.879
>50	38	30	8	
Gender				
M	51	41	10	0.357
F	9	6	3	
Differentiation degree				
Well	12	5	7	0.003*
Moderately	39	34	5	
Poorly	9	8	1	
Clinical stage				
I~II	28	19	9	0.065
III~IV	32	28	4	
Lymph node metastasis				
No	49	38	11	0.765
Yes	11	9	2	

Note: *P<0.05: HDAC1 (+) group vs HDAC1 (-) group.

Table 2. Correlation of clinicopathological characteristics with HDAC2 expression in HCC patients

Clinicopathological Characteristics	n	HDAC2 (+)	HDAC2 (-)	*P* value
Age (yr)				
≤50	22	17	5	0.503
>50	38	32	6	
Gender				
M	51	42	9	0.744
F	9	7	2	
Differentiation degree				
Well	12	9	3	0.714
Moderately	39	33	6	
Poorly	9	7	2	
Clinical stage				
I~II	28	21	7	0.212
III~IV	32	28	4	
Lymph node metastasis				
No	49	39	10	0.381
Yes	11	10	1	

(miR-29b, miR-34a, miR-449a and miR-520h) was detected in HCC tissues and adjacent normal tissues by qRT-PCR. As compared to adjacent normal tissues, the expression of 4 miRNAs reduced dramatically in HCC tissues ($P<0.05$; **Figure 2**).

According to the immunohistocehmical findings, patients were divided into HDAC1 (+) group and HDAC1 (-) group, and the expression of 4 miRNAs (miR-29b, miR-34a, miR-449a and miR-520h) was compared between them. Results showed the expression of 4 miRNAs in HDAC1 (+) group was significantly lower than in HDAC1 (-) group ($P<0.05$; **Figure 3**). This suggests that the HDAC1 expression is negatively related to the expression of 4 miRNAs.

microRNA acts on 3'UTR of HDAC1 mRNA to regulate HDAC1 expression

Analysis of bioinformatics databases (TargetScan and miRanda) showed miRNA could act on the 3'UTR of HDAC1 mRNA. Western blot assay indicated that HDAC1 expression reduced significantly after transfection with miR-34a mimic or miR-449a mimic. This indicates that miR-34a mimic and miR-449a mimic are able to reduce the HDAC1 expression in HCC.

HDAC1 mRNA with wild-type and mut-type 3'UTR was independent connected to pmirGLO, and miR-34a mimic (or miR-449a mimic) together with the vector was transfected into HepG2. The fluorescence signals were measured in these cells. Results showed, after co-transfection with miR-34a mimic and pmirGLO-HDAC1-wt, the luciferase activity reduced significantly as compared to control group (co-transfection with miR-NC and pmirGLO-HDAC1-wt) ($P<0.05$, **Figure 4E**, **4F**). After co-transfection with miR-34a mimic and pmirGLO-HDAC1-mut, the luciferase activity was comparable to that in control group (co-transfection with miR-NC and pmirGLO-HDAC1-mut) ($P>0.05$, **Figure 4E**, **4F**). This indicates that miR-34a may act on the seed region of 3'UTR of HDAC1 mRNA to negatively regulate its expression.

After co-transfection with miR-449a mimic and pmirGLO-HDAC1-wt, the luciferase activity reduced significantly as compared to control group (co-transfection with miR-NC and pmirGLO-HDAC1-wt) ($P<0.05$, **Figure 4E**, **4F**). After co-transfection with miR-449a mimic and pmirGLO-HDAC1-mut, the luciferase activity was similar to that in control group (co-transfection with miR-NC and pmirGLO-HDAC1-mut) ($P>0.05$, **Figure 4E**, **4F**). This indicates that miR-449a may act on the seed region of 3'UTR of HDAC1 mRNA to negatively regulate its expression.

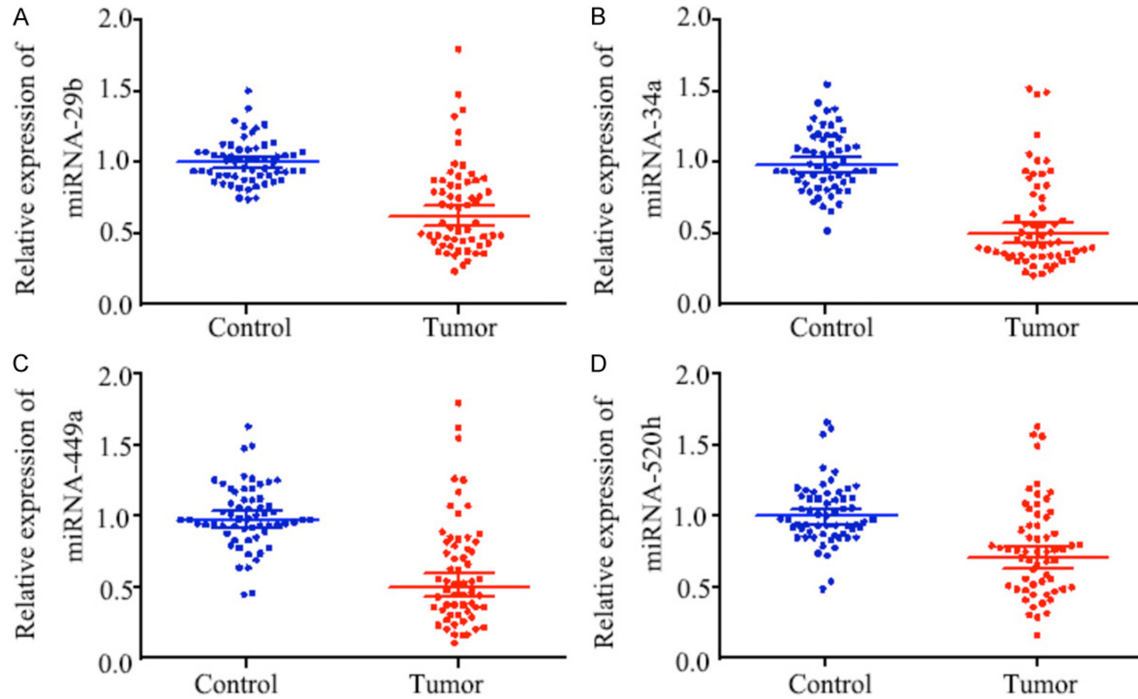

Figure 2. qRT-PCR is employed for the detection of expression of miR-29b, miR-34a, miR-449a and miR-520h in HCC tissues and adjacent normal tissues. A: miR-29b expression in HCC tissues is significantly lower than in adjacent normal tissues (P<0.05) (Tumor: HCC; Control: adjacent normal tissues); B: miR-34a expression in HCC tissues is significantly lower than in adjacent normal tissues (P<0.05) (Tumor: HCC; Control: adjacent normal tissues); C: miR-449a expression in HCC tissues is significantly lower than in adjacent normal tissues (P<0.05) (Tumor: HCC; Control: adjacent normal tissues); D: miR-520h expression in HCC tissues is significantly lower than in adjacent normal tissues (P<0.05) (Tumor: HCC; Control: adjacent normal tissues).

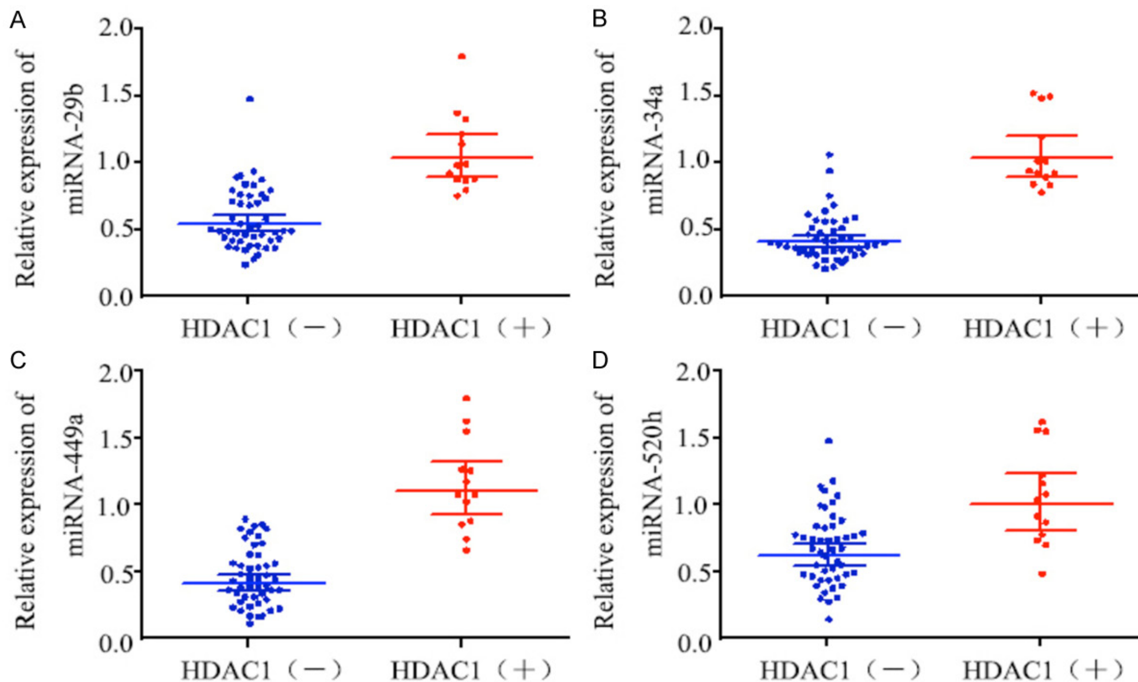

Figure 3. Expression of miR-29b, miR-34a, miR-449a and miR-520h in HDAC1 (+) group and HDAC1 (-) group. A: miR-29b expression in HDAC1 (+) group was markedly lower than in HDAC1 (-) group (*P*<0.05); B: miR-34a expres-

sion in HDAC1 (+) group was markedly lower than in HDAC1 (-) group ($P<0.05$); C: miR-449a expression in HDAC1 (+) group was markedly lower than in HDAC1 (-) group ($P<0.05$); D: miR-520h expression in HDAC1 (+) group was markedly lower than in HDAC1 (-) group ($P<0.05$).

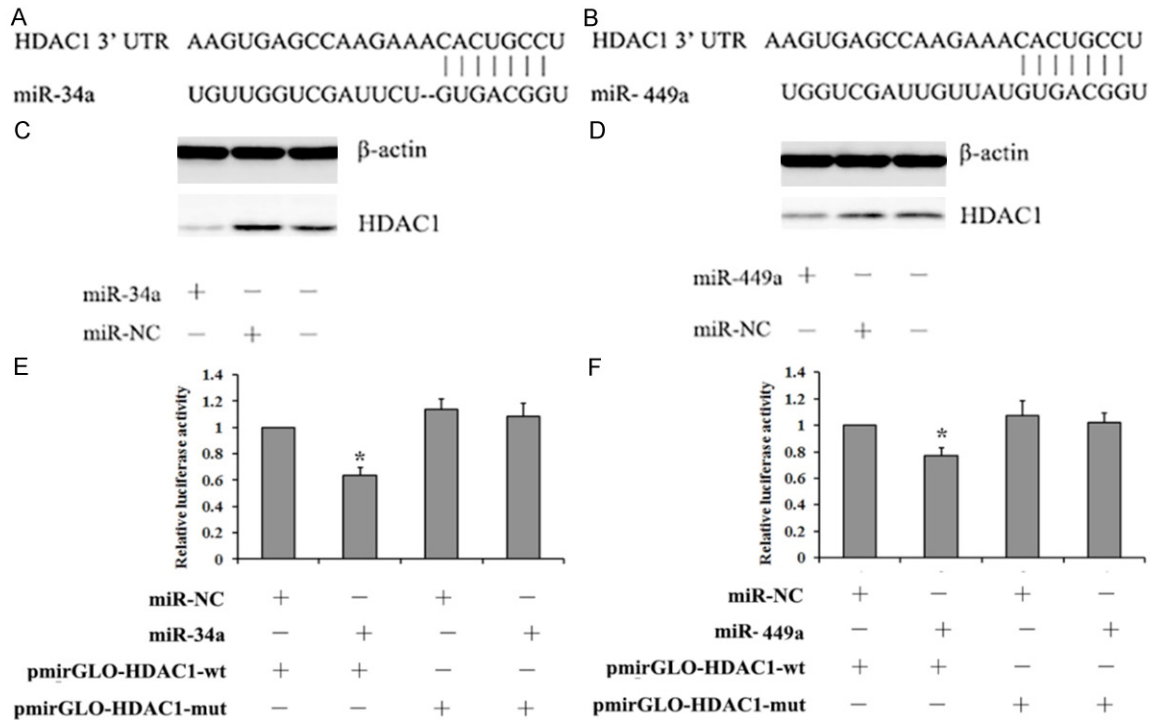

Figure 4. microRNAs act on the 3'UTR of HDAC1 mRNA to regulate its expression. A: miR-34a acts on the seed region of 3'UTR of HDAC1 mRNA. B: miR-449a acts on the seed region of 3'UTR of HDAC1 mRNA. C: Western blot assay showed miR-34a mimic was able to inhibit the HDAC1 protein expression in HepG2 cells; D: Western blot assay showed miR-449a mimic was able to inhibit the HDAC1 protein expression in HepG2 cells; E: Dual luciferase reporter assay showed, after co-transfection with miR-34a mimic and pmirGLO-HDAC1-wt, HepG2 cells had significantly reduced luciferase activity as compared to control group (co-transfection with miR-NC and pmirGLO-HDAC1-wt) ($P<0.05$); after co-transfection with miR-34a mimic and pmirGLO-HDAC1-mut, the luciferase activity in HepG2 cells was similar to that in control group (co-transfection with miR-NC and pmirGLO-HDAC1-mut) ($P>0.05$). F: Dual luciferase reporter assay showed, after co-transfection with miR-449a mimic and pmirGLO-HDAC1-wt, HepG2 cells had significantly reduced luciferase activity as compared to control group (co-transfection with miR-NC and pmirGLO-HDAC1-wt) ($P<0.05$); after co-transfection with miR-449a mimic and pmirGLO-HDAC1-mut, the luciferase activity in HepG2 cells was similar to that in control group (co-transfection with miR-NC and pmirGLO-HDAC1-mut) ($P>0.05$).

Discussion

It has been confirmed that epigenetic modifications including histone de-acetylation play important roles in the occurrence and development of malignancies in human [10-12]. In the occurrence and development of malignancies, to facilitate the histone de-acetylation may induce the transcription of some tumor suppressor genes related to the proliferation and metastasis of cancer cells, angiogenesis in the cancer and the differentiation and infiltration of cancer cells [13, 14], leading to the tumorogenesis. This may be one of mechanisms underlying the pathogenesis of cancers. In vitro and in vivo experiments have shown that to inhibit the HDACs expression may induce the cell cycle arrest in G2/M phase, inhibit the proliferation and infiltration of cancer cells and also promote the differentiation and apoptosis of cancer cells [15, 16].

Studies have revealed that histone acetylation is regulated by HDAC1 and HDAC2 [17, 18]. HDAC1 and HDAC2 may bind to the transcription complex to control the cancer growth. In the pancreatic cancer, ZEB1/HDAC transcription complex is able to inhibit the E-cadherin expression, leading to the metastasis of cancer cells [19]. In the present study, HDAC1 and

HDAC2 expression was detected in HCC tissues from 60 patients. Our results showed HDAC1 and HDAC2 expression was up-regulated in HCC tissues, suggesting that elevated HDACs expression may be related to the progression of HCC. Further analysis showed HDAC1 expression in well to moderately differentiated HCC was significantly lower than in poorly differentiated HCC (P<0.05), which was consistent with previously reported [20]. Thus, to reduce HDACs expression has the potential to inhibit the cancer growth. There is evidence showing that silencing of HDAC1 via RNA interfering may inhibit the progression of some hematological malignancies and solid cancers via arresting cell cycle, inducing the differentiation of cancer cells and facilitating the apoptosis of cancer cells [21, 22].

In recent years, numerous studies have been conducted to investigate the miRNAs related to HCC [23]. Studies reveal that miRNA may regulate target genes to play important roles in the occurrence and development of HCC [24, 25]. Our results showed the HDAC1 expression increased significantly. As an important gene regulator, miRNA may act on target genes to result in abnormal HDAC1 expression in HCC [26]. After bioinformatics analysis, miRNAs targeting HDAC1 were screened, of which miR-29b, miR-34a, miR-449a and miR-520h were further investigated. qRT-PCR showed the expression of 4 miRNAs in HCC tissues was markedly lower than in adjacent normal tissues. In HCC tissues, HDAC1 expression was negatively related to the expression of miR-29b, miR-34a, miR-449a and miR-520h. Thus, miRNA may act on HDAC1 to regulate its expression, which plays important roles in the occurrence and development of HCC.

To further explore the effect of miRNA on HDAC1, the miR-34a and miR-449a were further investigated in this study. HDAC1 3'UTR was cloned into dual luciferase reporter vector pmirGLO. Then, miR-34a mimic or miR-449a mimic together with the vector was co-transfected into HCC cells. Results showed miR-34a or miR-449a was able to bind to the seed region of 3'UTR of HDAC1 mRNA to negatively regulate its expression. Thus, miRNAs may become a target in the therapy of HCC with abnormal HDAC1 expression.

This study indicates that the HDACs expression is abnormal in HCC, and the abnormal HDACs expression is closely associated with the occurrence and development of cancers. Several miRNAs have been found to regulate the HDAC1 expression. Thus, HDAC1 over-expression in cancers may result from multiple factors, and miRNAs may become a target in the molecular therapy of HCC with abnormal HDAC1 expression based on the fact that miRNAs are able to regulate HDAC1 expression.

Disclosure of conflict of interest

None.

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