The Histone Deacetylase 4/Sp1/microRNA-200a Regulatory Network Contributes to Aberrant Histone Acetylation in Hepatocellular Carcinoma

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As an important epigenetic mechanism, histone acetylation modulates the transcription of many genes and plays important roles in hepatocellular carcinoma (HCC). Aberrations in histone acetylation have been observed in HCC, but the factors that contribute to the aberrations have not been fully elucidated. MicroRNAs (miRNAs), which are noncoding RNAs that regulate gene expression, are involved in important epigenetic mechanisms. In this study, we determined that miR-200a and the level of histone H3 acetylation at its promoter were reduced in human HCC tissues in comparison with adjacent noncancerous hepatic tissues. Furthermore, our results suggested that the histone deacetylase 4 (HDAC4) inhibited the expression of miR-200a and its promoter activity and reduced the histone H3 acetylation level at the mir-200a promoter through a Sp1-dependent pathway. Interestingly, we observed that the miR-200a directly targeted the 3'-untranslated region of the HDAC4 messenger RNA and repressed expression of HDAC4. Therefore, miR-200a ultimately induced its own transcription and increased the histone H3 acetylation level at its own promoter. Through targeting HDAC4, miR-200a also induced the up-regulation of total acetyl-histone H3 levels and increased the histone H3 acetylation level at the p21WAF/Cip1 promoter. Finally, we determined that miR-200a inhibited the proliferation and migration of HCC cells in vivo and in vitro. Conclusion: Our findings suggest that the HDAC4/Sp1/miR-200a regulatory network induces the down-regulation of miR-200a and the up-regulation of HDAC4 in HCC. As a result, down-regulation of miR-200a enhances the proliferation and migration of HCC cells and induces aberrant histone acetylation in HCC. These findings highlight a potential therapeutic approach in targeting the HDAC4/Sp1/miR-200a regulatory network for the treatment of HCC. (HEPATOLOGY 2011;54:2025-2035)
acetyl groups from histones, HDACs regulate the expression of numerous proteins involved in both cancer initiation and cancer progression.\textsuperscript{8,9} The aberrant expression of HDACs and the aberrant regulation of histone acetylation have been observed in various types of cancer, including HCC.\textsuperscript{10,11} However, the mechanism responsible for the aberrations has not been fully elucidated.

MicroRNAs are evolutionarily conserved noncoding RNAs with lengths of 21-25 nucleotides, which play critical roles in the regulation of gene expression and multiple cellular processes.\textsuperscript{12,13} Through base pairing with messenger RNAs (mRNAs) at partially or fully complementary sites, miRNAs induce mRNA cleavage or translational repression.\textsuperscript{14} A growing body of evidence supports a role for miRNAs as both targets and effectors of aberrant histone acetylation. The expression of many miRNAs has been indicated to be affected by HDAC inhibitors.\textsuperscript{15-17} Several miRNAs have been reported to target the histone acetylation modification enzymes.\textsuperscript{18-20} However, whether the alterations in the expression of miRNAs induced by HDAC inhibitors are due to changes in histone acetylation levels at their promoters remains to be investigated. Also, whether miRNAs regulate global histone acetylation levels or histone acetylation modifications at particular sites through the targeting of histone acetylation modification enzymes has not been reported in the context of HCC.

Our previous studies\textsuperscript{21} indicate that the expression of microRNA-200a (miR-200a) was down-regulated in the livers of HBX transgenic mice, which were prone to develop HCC, in comparison with the livers of wild-type mice. In this study, we observed that the expression of the miR-200a was down-regulated in human HCC tissues in comparison with the adjacent noncancerous hepatic tissues. Intriguingly, the histone H3 acetylation level at the \textit{mir-200a} promoter was also down-regulated in human HCC samples. Further analysis demonstrated that HDAC4/Sp1 contributed to the down-regulation of miR-200a through the deacetylation of histone H3 at its promoter. We also determined that miR-200a repressed HDAC4 expression. Therefore, miR-200a ultimately increased its own transcription. Through targeting HDAC4, miR-200a increased the global level of acetyl-histone H3 and induced aberrant histone acetylation at its own promoter and the \textit{p21^{WAF/Cip1}} promoter.

Materials and Methods

For a description of the materials and methods used in this study, see the Supporting Information.

Results

\textbf{The miR-200a Is Down-Regulated in Human HCC Tissues.} To determine whether the miR-200a was differentially expressed in human primary liver cancer, the expression level of miR-200a was examined using real-time polymerase chain reaction (PCR) in 41 pairs of human HCC tissues and pair-matched adjacent noncancerous hepatic tissues. The expression of the miR-200a was analyzed by real-time PCR and normalized to U6. The results are displayed on a log scale. The statistical differences between samples were analyzed with the Wilcoxon signed-rank test (\(n = 41, P < 0.01\)).

\textbf{HDAC4/Sp1 Inhibits Expression of miR-200a.} To determine how transcription of \textit{mir-200a} was controlled, we investigated whether DNA methylation may contribute to the down-regulation of the miR-200a. We identified a 2500–base pair cytosine–guanine dinucleotide (CpG) island in the \textit{mir-200a} promoter, just as in other reports.\textsuperscript{22} We performed bisulfite sequencing analysis in five pairs of human tissue samples from Fig. 1 in which the miR-200a level decreased more than 90% as compared with matched controls. We found these regions hypermethylated in
both HCC and matched controls (Supporting Fig. 1),
thus indicating DNA methylation is less likely to regu-
late miR-200a expression in HCC.

Next, we examined the genomic sequences in the 5' direction of the mir-200a gene.23,24 Intriguingly, mul-
tiple binding sites were observed for Sp1 transcription
factors. Recent studies have validated that HDAC4
inhibits the expression of several genes and induces
histone deacetylation through Sp1 binding sites.25,26
We tested whether HDAC4 could induce histone H3
hypoacetylation of the mir-200a promoter and con-
tribute to the down-regulation of miR-200a expres-
sion. We enhanced HDAC4 expression by transfecting
an HDAC4 expression vector (pcDNA3.1-HDAC4)
into SMMC-7721 and HepG2 cells and employing
the pcDNA3.1 vector as the negative control (Fig.
2A), and we inhibited HDAC4 expression by trans-
flecting HDAC4 small interfering RNA (siRNA) into
SMMC-7721 and HepG2 cells with control siRNA as
the negative control (Fig. 2B). After 48 hours of trans-
fection, we measured the expression level of miR-200a.
Our results indicated that enforced HDAC4 expression
decreased miR-200a level (Fig. 2C). The inhibition of
HDAC4 increased the expression of miR-200a in a
corresponding manner (Fig. 2D). Nevertheless, we first
inhibited Sp1 expression by transfecting Sp1 siRNA
(Fig. 2E), and we induced or inhibited HDAC4
expression 24 hours later. We measured the expression

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**Fig. 2. Effects of HDAC4 on the expression of miR-200a.** (A) HDAC4 protein levels after the transfection of the HDAC4 expression vector
(pcDNA3.1-HDAC4) or the control vector into SMMC-7721 and HepG2 cells. (B) HDAC4 protein levels after the transfection of HDAC4 siRNA or
the control siRNA into SMMC-7721 and HepG2 cells. (C) Expression level of miR-200a after the transfection of pcDNA3.1-HDAC4 or pcDNA3.1
into SMMC-7721 and HepG2 cells. (D) Expression level of miR-200a after the transfection of HDAC4 siRNA or the control siRNA into SMMC-
7721 and HepG2 cells. (E) Sp1 mRNA levels after the transfection of Sp1 siRNA or the control siRNA into SMMC-7721 cells. (F) Expression
level of miR-200a after the transfection of pcDNA3.1-HDAC4, pcDNA3.1, HDAC4 siRNA, or the control siRNA into SMMC-7721 cells in which
Sp1 had been silenced by siRNA 24 hours previously. Data are presented as the mean ± standard error, based on at least three independent
experiments. *P < 0.05, **P < 0.01.
level of miR-200a 48 hours later and determined that HDAC4 could not inhibit the expression of miR-200a (Fig. 2F). In addition to miR-200a, the miR-200 family also contains miR-141, miR-200b, miR-200c, and miR-429. We first measured the expression of these miRNAs in SMMC-7721 and HepG2 cells and found that the expressions of miR-200a, miR-200b, and miR-429 are higher than that of miR-200c and miR-141 (Supporting Fig. 2A). After the enhancement or inhibition of HDAC4 expression in SMMC-7721 and HepG2 cells, we tested the expression of the miRNAs and found that enforced HDAC4 expression also decreased levels of miR-200b and miR-429 (Supporting Fig. 2B). The inhibition of HDAC4 increased the expression of miR-200b and miR-429 (Supporting Fig. 2C). Expression of miR-141 and miR-200c did not change upon the enhancement or inhibition of HDAC4 expression (Supporting Fig. 2B,C).

To further examine the role of HDAC4 on miR-200a, we cloned the promoter of the mir-200a gene from −965 to +193 base pairs upstream of the transcription start site into the pGL3 basic firefly luciferase reporter and cotransfected the construct with pcDNA3.1-HDAC4 or HDAC4 siRNA into the SMMC-7721 cells. The pGL3 basic firefly luciferase reporter was used as a negative control. The p21WAF/Cip1 promoter subcloned into the same vector was used as a positive control. HDAC4 significantly reduced the luciferase activity of the construct, and inhibition of HDAC4 increased the luciferase activity of the construct, which were similar to the effect on the p21WAF/Cip1 promoter (Fig. 3A,B). We then mutated the Sp1 recognition sites (Fig. 3C) and cotransfected cells with pcDNA3.1-HDAC4. Our results indicated that the mutation of Sp1 recognition sites attenuated the effects of HDAC4 on the promoter activity of mir-200a (Fig. 3D).

We analyzed, through chromatin immunoprecipitation (ChIP) assays, the effects of HDAC4 on the histone H3 acetylation level at the Sp1 recognition site-rich region of the mir-200a promoter. Ectopic HDAC4 expression significantly decreased the histone H3 acetylation level at the mir-200a promoter (Fig. 3E). Together, these results suggest that HDAC4 inhibits the expression of miR-200a and its promoter activity and reduces the histone H3 acetylation level at the mir-200a promoter through a Sp1-dependent pathway.

**HDAC4 Expression Is Inversely Correlated with miR-200a in Human HCC Tissues.** Because HDAC4 could repress the expression of miR-200a, we investigated whether an inverse relationship exists between HDAC4 expression and levels of miR-200a. We examined expression of HDAC4 mRNA in human tissue samples from Fig. 1. The HDAC4 mRNA levels were significantly up-regulated in HCC samples in comparison with adjacent noncancerous liver tissues ($P < 0.01$, Wilcoxon signed-rank test; Fig. 4A). Next, we investigated whether HDAC4 mRNA expression was inversely correlated with levels of miR-200a in HCC tissues. A total of 41 HCCs were analyzed for the expression levels of HDAC4 mRNAs and for miR-200a expression by real-time PCR. A statistically significant inverse correlation was observed between HDAC4 mRNA and miR-200a ($n = 41$, $r = −0.375$, $P = 0.016$, Pearson's correlation; Fig. 4B), supporting the role of HDAC4 in the expression of miR-200a.

**Histone H3 Acetylation Level of the mir-200a Promoter Is Down-Regulated in Human HCC Tissues.** To determine whether down-regulation of miR-200a in human primary liver cancer was due to the decreased acetylation level of histone H3 at the mir-200a promoter, we used ChIP assays to measure histone H3 acetylation levels at the mir-200a promoter in six randomly selected pairs of human tissue samples from Fig. 1. Histone H3 acetylation levels were significantly decreased in five of the six HCC samples in comparison with the adjacent noncancerous hepatic tissues (Fig. 4C). Interestingly, we found that the histone H3 acetylation level was correlated with miR-200a expression level in all of the six samples tested and was inversely correlated with HDAC4 in five of the six samples tested (indicated by asterisks, Fig. 4C). These data suggested that down-regulation of miR-200a was at least partially due to the reduced histone H3 acetylation level at the mir-200a promoter caused by HDAC4.

**miR-200a Represses HDAC4 Expression.** Because HDAC4 was overexpressed in HCC and was inversely correlated with miR-200a expression, we wondered whether the increase of HDAC4 expression could be driven by the reduction of miR-200a expression. We performed an online search of the TargetScan and found that miR-200a could bind to the 3′-untranslated region (UTR) of the human HDAC4 mRNA at two potential target sites that are partially complementary to miR-200a (Fig. 4A). To validate the interactions between miRNA and target, these two HDAC4 complementary sites were individually cloned into the 3′-UTR of the firefly luciferase gene and cotransfected with miR-200a mimics or miRNA negative control into SMMC-7721 cells. The pMIR-REPORT vector was used as a negative control. The 3′-UTR of SIP1 subcloned into the same vector was used as a positive
miR-200a significantly reduced the luciferase activity of the first construct of the HDAC4 3′-UTR with respect to the miRNA negative control, which was similar to the effect on the SIP1 3′-UTR report. However, miR-200a did not reduce the luciferase activity of the second construct (Fig. 5B), indicating that miR-200a may exert its effect on HDAC4 primarily through the first target site. Next, we measured the
mRNA and protein levels of HDAC4 in SMMC-7721 and HepG2 cells with miR-200a mimics or the miRNA negative control transfection or with the miR-200a inhibitor or miRNA inhibitor negative control transfection through reverse-transcription PCR (RT-PCR) and western blotting. Neither induction of expression nor the inhibition of miR-200a could change HDAC4 mRNA levels (Fig. 5C,D). However, enforced miR-200a expression led to a reduction of HDAC4 protein levels in comparison with the negative control in the two human HCC cell lines (Fig. 5E). On the contrary, the inhibition of miR-200a increased the HDAC4 protein levels (Fig. 5F). These results strongly indicated that the expression of the HDAC4 gene was translationally suppressed directly by miR-200a. We next tested whether other miR-200 family members could change HDAC4 expression. Similarly we transfected mimics of the other miR-200 family members into SMMC-7721 and HepG2 cells and measured the protein levels of HDAC4. Our results showed that miR-141 could reduce the protein level of HDAC4, whereas miR-200b, miR-200c, and miR-429 could not change the protein level of HDAC4 (Supporting Fig. 3).

**The Autoregulatory Loop of miR-200a.** Having demonstrated that HDAC4 repressed the transcription of miR-200a and decreased the histone H3 acetylation level at the mir-200a promoter and that miR-200a reduced the expression of HDAC4, we next investigated whether aberrant expression of miR-200a may “feed back” to regulate its own transcription and the histone H3 acetylation level at its promoter through the HDAC4/Sp1/mir-200a network. The miR-200a-promoter reporter construct was cotransfected with miR-200a mimics or the negative control into SMMC-7721 cells. miR-200a significantly increased...
the luciferase activity of the construct to approximately 2.5-fold \( (P = 0.004) \) in comparison with the negative control (Fig. 6A). Conversely, when we cotransfected the construct and pcDNA3.1-HDAC4, which does not contain the miR-200a binding site and cannot be inhibited by miR-200a, with the miR-200a mimics or scrambled oligonucleotides as the miRNA negative control, HDAC4 mRNA expression levels after the transfection of miR-200a mimics or the miRNA negative control (NC) into HepG2 and SMMC-7721 cells. HDAC4 mRNA expression levels after the transfection of the miR-200a inhibitor or miRNA inhibitor negative control (NC) into HepG2 and SMMC-7721 cells. Expression of the HDAC4 protein after the transfection of the miR-200a inhibitor or the negative control (NC) into HepG2 and SMMC-7721 cells. (Left panel) Western blots indicating approximately 140 kDa HDAC4 in HCC cell lines. (Right panel) The intensities of the bands from the western blots were quantified by densitometry. Data are presented as the mean ± standard error, based on at least three independent experiments. \*\( P < 0.05 \).

**Overexpression of miR-200a Induces Up-Regulation of Global Acetyl-Histone H3 and Local Histone H3 Acetylation Level at the p21WAF1/Cip1 Promoter.** We transfected pcDNA3.1-HDAC4 or pcDNA3.1 as the negative control into HepG2 cells, and 48 hours later, we examined acetyl-histone H3 by western blotting. The ectopic expression of HDAC4 significantly reduced global acetyl-histone H3 (Fig. 6D). Next, we transfected miR-200a mimics or the miRNA negative control into HepG2 cells, and 48 hours later, we examined global acetyl-histone H3 by western blotting. Our result demonstrated that miR-200a up-regulated global acetyl-histone H3 (Fig. 6E).

Recent studies have indicated that HDAC4 deacetylated histone H3 at the p21WAF1/Cip1 promoter region. Now that miR-200a could inhibit HDAC4 expression, we assessed, through ChIP assays, whether overexpression of miR-200a could increase histone H3 acetylation level at the p21WAF1/Cip1 promoter. Our results indicate that ectopic expression of miR-200a...
significantly increases the histone H3 acetylation level at the p21WAF/Cip1 promoter (Fig. 6F). These results demonstrate that miR-200a induced aberrant histone acetylation in HCC by targeting HDAC4.

miR-200a Inhibits the Proliferation and Migration of HCC Cells In Vitro and In Vivo. To investigate the biological effects of miR-200a on human HCC, we generated two stably transfected cell lines containing integrated copies of miR-200a or a control lentiviral expression vector. We observed significant up-regulation of miR-200a in the stably transfected cell lines compared with cells transfected with negative control (Fig. 7A). Overexpression of miR-200a inhibited cell proliferation (Fig. 7B) and migration (Fig. 7C,D) in vitro. The stably transfected cells were implanted subcutaneously into the flanks of nude mice. Up-regulation of miR-200a significantly decreased overall tumor growth, as assessed by measurements of tumor volume (Fig. 7E,F).

Discussion

The aberrant histone acetylation at the promoters of cellular genes is an important feature in the development of human cancers.30,31 Many tumor suppressor genes, such as p21WAF/Cip1 and TMS1 (target of methylation-induced silencing 1), have been demonstrated to be silenced by promoter hypoacetylation.26,32 The global inhibition of HDAC activity has been indicated to stimulate antitumor effects, and the approval of the HDAC inhibitor suberoylanilide hydroxamic acid by the US Food and Drug Administration for the treatment of cutaneous T cell lymphoma, validates the importance of histone acetylation in carcinogenesis.33,34 However, the mechanism responsible for aberrations in histone acetylation remains largely unknown.

In this study, for the first time, we identified miR-200a as both the target and the effector of aberrant histone acetylation in HCC. HDAC4 induced the histone H3 deacetylation of the miR-200a promoter, repressed the transcription of miR-200a, and finally, repressed the expression of miR-200a through a Sp1-dependent pathway. Our data demonstrate that miR-200a is frequently down-regulated in HCC tissues in comparison with the adjacent noncancerous hepatic tissues, a finding that is consistent with other reports.35,36 Reduced levels of the histone H3 acetylation at the miR-200a promoter and increased levels of HDAC4 mRNA were also observed in HCCs. Because HDAC4 alone is enzymatically inactive, it may suppress the transcription of miR-200a and induce the histone H3 deacetylation at the miR-200a
promoter by recruiting catalytically active HDACs into transcriptional corepressor complexes. Therefore, further investigations are required to fully elucidate the nature of HDAC4-containing repressor complexes at the mir-200a promoter.

In addition to miR-200a, the miR-200 family also includes miR-200b, miR-200c, miR-141, and miR-429, with miR-200b, miR-200a, and miR-429 being located on chromosome 1 and miR-200c and miR-141 being located on chromosome 12. Both clusters are encoded as polycistronic transcripts. Our results show that HDAC4 regulates the expression of the miR-200b, miR-200a, and miR-429 cluster, but does not regulate the other cluster. Other reports have demonstrated that HDAC inhibitors induce up-regulation of miR-200c, and therefore we speculated that other HDACs may participate in the regulation of the miR-200c and miR-141 cluster.

Interestingly, we observed that miR-200a, in turn, negatively regulated HDAC4 expression by directly targeting the complementary sites in the 3' UTR of HDAC4 mRNA, generating a double negative feedback loop. Feedback loops are common in many genetic pathways involving miRNAs, and they seem to enhance the robustness of gene networks. A significant inverse correlation was also observed between HDAC4 and miR-200a in human HCC tissues. Copy number alterations of miR-200a and HDAC4 were not found in HCC tissues compared with matched controls. Other proteins such as ZEB1, SIRT1, p53, and gata-binding factors can also regulate the expression of miR-200a. Therefore, there is an intricate

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**Fig. 7. Biological effects of miR-200a in HCC cells.** (A) Expression level of miR-200a in stably transfected cell lines. The expression of miR-200a was analyzed by real-time PCR and normalized to U6. Data are presented as the mean ± standard error, based on at least three independent experiments. **P < 0.01. miR NC, miR-200a negative control. (B) miR-200a inhibits the proliferation of HepG2 and SMMC-7721 cells. Cell number was determined by the CCK-8 assay, and the relative number of cells is presented (mean ± standard error). All of the assays were performed in triplicate and repeated at least three times. **P < 0.01. (C) Determination of miR-200a involvement in cell migration by transwell assays. Stably transfected HepG2 or SMMC-7721 cells (40,000) were plated in medium without serum in the upper chamber of a transwell. Medium in the lower chamber with 10% serum was employed as a chemoattractant. Cells were incubated for 24 hours, and those cells that did not migrate through the pores were removed with a cotton swab. Cells on the underside of the culture insert were stained with crystal violet and counted under the microscope. The images are representative of at least three independent experiments employing SMMC-7721 cells. (D) The relative level of cell migration is presented as the mean ± standard error, based on at least three independent experiments. **P < 0.05, **P < 0.01. (E) The effects of miR-200a on HCC tumor growth in subcutaneous models. (F) An in vivo subcutaneous tumor growth curve is shown for HepG2 cells stably transfected with miR-200a or control (miR NC) (n = 5, **P < 0.01).
mechanism regulating the expression of miR-200a and HDAC4 in HCCs. Further investigations are required to elucidate whether the up-regulation of HDAC4 or the down-regulation of miR-200a is the initial factor of this loop in HCC.

Recently, many studies have demonstrated that miRNAs may affect the epigenetic mechanism. For instance, miR-152 induced aberrant DNA methylation in HCC by targeting the DNA methyltransferase 1, as demonstrated in our previous study.\(^4\) Other miRNAs, such as miR-148a/b,\(^6\) miR-1,\(^20\) and miR-449a,\(^15\) have also been reported to target epigenetic modifying enzymes and modulate the epigenetic transcriptional-regulatory process. However, whether miRNAs can affect the histone acetylation level in HCC remains largely unknown. To our knowledge, this report is the first to show that miR-200a induces up-regulation of the total acetyl-histone H3 level and increases the local histone H3 acetylation level at the p21\(^{\text{WAF1/Cip1}}\) promoter in HCC. While our manuscript was under review, Eades et al. showed that miR-200a targeted a class III histone deacetylase (SIRT1) and damaged the recruitment of DNA methyltransferase to tumor suppressor genes.\(^{22}\) This extended the role of miR-200a as an important epigenetic modification modulator, in that it could not only change histone acetylation level, but also could change DNA methylation level.

The ectopic expression of miR-200a in HCC cells causes the inhibition of cell proliferation and migration. This finding indicates that miR-200a functioned as a tumor suppressor gene, which was also supported by the down-regulation of miR-200a observed in HCCs. These results demonstrate that the enhanced expression of the miR-200a by gene transfer can reverse the malignant phenotypes of HCC cells and suggested that miR-200a represents a potential therapeutic target of HCC.

Collectively, our studies identified the interesting HDAC4/Spl/miR-200a regulatory network, which contributes to the down-regulation of miR-200a, the up-regulation of HDAC4, and the aberrant histone acetylation in HCC. We determined that down-regulation of miR-200a is an important contributor to proliferation and migration of HCC cells. We believe that synthetic miR-200a, alone or with specific HDAC4 inhibitors, represents a potential strategy for the treatment of HCC.

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