MicroRNA-138 inhibits cell proliferation in hepatocellular carcinoma by targeting Sirt1

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Abstract. MicroRNAs (miRNAs) are a family of small, non-coding RNA molecules that are highly conserved across species and function as regulators of gene expression. In the present study, we revealed that miR-138 expression was at a low level while sirtuin type 1 (Sirt1) mRNA expression was at high level in hepatocellular carcinoma tissues and cell lines by using real-time PCR and western blot assays, and the functions of miR-138 were achieved via targeting of Sirt1 using luciferase reporter gene vector and RNA immunoprecipitation assays. Overexpression of miR-138 attenuated Sirt1 expression and inhibited cell proliferation by using CCK-8 and BrdU assays. The inhibitory effect of miR-138 could be partially restored by forced expression of Sirt1 in cells. Our data revealed a crucial role and mechanism of miR-138 in the regulation of hepatocellular carcinoma cell growth via the miR-138/Sirt1 axis, and miR-138 could be an important potential target for the clinical management of hepatocellular carcinoma in the future.

Introduction

In the past few years, the prognosis for hepatocellular carcinoma patients has improved through treatment with a combination of chemotherapy and aggressive surgical resection. However, patients with local relapse or distant metastasis have a poor prognosis (1-3). Hence, searching for brand-new approaches to treat relapsing and/or metastatic hepatocellular carcinoma has become essential. Recently, more and more studies have reported that microRNAs (miRNAs) are closely associated with cancer. miRNAs are a recently discovered series of non-coding small RNAs that exert their functions via regulation of diverse gene expression. Mature miRNAs achieve their functions via merging with an RNA-inducing silencing complex (RISC) and binding to respective complementary sites within the 3' untranslated region (3'UTR) of the mRNA of the specific target genes, thus hindering translation or directly inducing degradation (4-7).

miRNA expression significantly varies with various types of cancers. In addition, it could potentially be a notable diagnostic and/or prognostic tool (8). It is important to clarify the effect of miRNAs on the pathogenic mechanisms and progression of tumors as a result of miRNAs feasible regulation of various critical biological processes, including the differentiation, progression, apoptosis, proliferation and sensitivity to treatment of tumor cells (9). However, the expression and dysregulation of miRNAs in hepatocellular carcinoma are still unclear and hepatocellular carcinoma exhibits a poor prognosis and high possibility of tumor proliferation and migration. Although results from numerous studies have identified proliferation and migration as the causes of death from solid tumors, not much was known concerning the molecular mechanism underlying them.

Bioinformatic algorithms estimate that up to 30% of human genes are modulated by all the human miRNAs, which may affect most genetic pathways (10). Numerous studies have clarified specific miRNA expression profiles of a variety of cancer tissues compared to those of normal adjacent tissues. miRNAs may have either a suppressive or promotive effect on tumors via the cellular microenvironment and targeting the genes that they regulate (11,12). According to previous studies, miR-138 was confirmed to be significantly downregulated in various types of cancer and play a key role as a tumor suppressor among these functional miRNAs (13). For example, miR-138 suppresses nasopharyngeal carcinoma proliferation and migration (14). miR-138 inhibits tumor growth through suppression of EZH2 in non-small cell lung cancer (15). miR-138 inhibits the proliferation of non-small cell lung cancer cells by targeting 3-phosphoinositide-dependent protein kinase-1 (16). Moreover, one study found that miR-138 was downregulated in 77.8% (14/18) of HCC tissues compared with adjacent non-tumor tissues, which demonstrated the involvement of miR-138 in hepatocellular carcinoma proliferation and metastasis (17). However, the biological role and mechanism of miR-138 in hepatocellular carcinoma have yet to be reported. These aforementioned studies inspired us to investigate the detailed functions and mechanism of miR-138, a well-established tumor-suppressor miRNA, in HCC.

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Sirtuins are a series of class III histone deacetylases that are dependent on nicotinamide adenine dinucleotide (NAD^+) and are conserved across species. There are seven members in the mammalian sirtuin family, including sirtuin type 1 (SIRT1) to SIRT7. They are characterized by a conserved 275-amino acid catalytic core and specifically added N-terminal and/or C-terminal sequences of diverse lengths (18). Among all the members, SIRT1 is the most studied sirtuin. Studies have reported that it has >10 substrates, including Ku70, p53, NF-κB and forkhead transcription factors (FOXOs). Through cooperation with different substrates, SIRT1 responds to stress and DNA damage, and also affects cellular responses to DNA damage (19).

In the present study, we revealed the regulatory association between miR-138, known as a tumor suppressor, and Sirt1, known as an oncogene. We demonstrated that miR-138 suppressed the proliferation and migration of hepatocellular carcinoma cells, most likely by targeting Sirt1.

Materials and methods

Tissue samples, cell lines and cell transfection. We obtained a total of 37 pairs of primary hepatocellular carcinoma and their matched adjacent normal tissues from patients who underwent surgical resections at Xiangya Hospital of Central South University (Changsha, China). All samples were snap-frozen in liquid nitrogen, and then stored at -80˚C until further use. This project was approved by the Ethics Committee of Xiangya Hospital of Central South University. All patients' informed consents were obtained.

We purchased the human hepatocellular cell line L02 and four human hepatocellular carcinoma cell lines, including HepG2, SMMC7721, Bel7404 and HCCM3 from the American Type Culture Collection (ATCC; Manassas, VA, USA). We routinely cultured cells in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco, CA, USA) and incubation followed at 37˚C in a humidified atmosphere of 5% CO_2. By transfection with miR-138 mimics we achieved ectopic expression of miR-138 in cells (GenePharma, Shanghai, China) using Lipofectamine 2000 (Invitrogen). We performed overexpression of Sirt1 using Sirt1 ORF expression clone (GeneCopoecia, Guangzhou, China). Then, we plated cells in 6-well clusters or 96-well plates and transfected them for 24 or 48 h. Transfected cells were used in further assays or RNA/protein extraction.

RNA extraction and SYBR-Green quantitative PCR analysis. We extracted total RNA from cells using TRIzol reagent (Invitrogen), and detected mature miR-138 expression in cells using a Hairpin-it™ miRNAs qPCR Quantitation kit (GenePharma). We used the expression of RNU6B and L02 cells as endogenous controls and used SYBR-Green qPCR assay (Takara, Dalian, China) to assess the expression of Sirt1. The $2^{-\Delta\Delta Ct}$ method was used to process the data.

CCK-8 cell proliferation assay. Cell Counting Kit-8 (CCK-8) (Beyotime, Hangzhou, China) was used to assess cell proliferation rates. We seeded $0.5x10^4$ cells/well in a 96-well plate for 24 h, transfected them with the indicated miRNA or siRNA, and further incubated the cells for 24, 48, 72 and 96 h, respectively. One hour before the endpoint of incubation we added 10 μl of CCK-8 reagent to each well. A microplate reader was used to determine the OD_{450nm} value in each well.

Western blot analysis. The expression of Sirt1 and epidermal growth factor receptor (EGFR) in hepatocellular carcinoma cell lines was detected by performing immunoblotting. We lysed, cultured or transfected cells in RIPA buffer with 1% phenylmethylsulfonyl fluoride (PMSF) and loaded protein onto an SDS-PAGE minigel and then transferred the protein onto a polyvinylidene fluoride (PVDF) membrane. We probed the blots with 1:1,000 diluted rabbit polyclonal Sirt1 antibody (Abcam, Cambridge, MA, USA) at 4˚C overnight, and then incubated them with an HRP-conjugated secondary antibody (1:5,000). Subsequently, enhanced chemiluminescence (ECL) substrates (Millipore, Billerica, MA, USA) were used to visualize the signals. We used β-actin as an endogenous protein for normalization.

Luciferase reporter assay. PCR was performed to amplify a fragment of the 3'UTR of Sirt1 (1,089 bp) containing the putative miR-138 binding site (1510-1517) using the following primers: wt-Sirt1 (forward) 5'-CCGCTCGAGCACCAGTAAAACAAGGAACTTG-3' and wt-Sirt1 (reverse) 5'-GAATGCGGCCGCTTTACAGAAACAAATGCAATGTTAC-3'. Then, we subcloned the PCR product into a psiCHECK-2 vector (Promega, Madison, WI, USA) immediately downstream to the luciferase gene sequence. We also synthesized a psiCHECK-2 construct containing the 3'UTR of Sirt1 with a mutant seed sequence of miR-138 using the following primers: mut-Sirt1 (forward) 5'-TTAAAATTTCCTACTTGTGTATAGAAATGGAAAG-3 and mut-Sirt1 (reverse) 5'-ACAAGTAGGAAATTTTAATACAGTGGTTCTC-3'.

DNA sequencing was used to verify all constructs. HepG2 and SMMC7721 cells were plated into 96-well clusters, then co-transfected with 100 ng of constructs with or without miR-138 precursors. At 48 h after transfection, a Dual-Luciferase Reporter Assay System (Promega) was used to detect luciferase activity. In addition, luciferase activity was normalized to *Renilla* activity.

RNA immunoprecipitation. RNA immunoprecipitation assays were performed using the Imprint RNA Immunoprecipitation kit (Sigma-Aldrich, St. Louis, MO, USA) along with the AGO2 antibody (Cell Signaling, Rockford, IL, USA). The AGO2 antibody was then recovered by protein A/G beads. Sirt1 and miR-138 RNA levels in the immunoprecipitates were assessed by qRT-PCR.

Statistical analysis. All data were obtained from three independent experiments and are expressed as the mean ± SD and processed using SPSS 17.0 statistical software (SPSS, Inc., Chicago, IL, USA). We compared the expression of miR-138 in hepatocellular carcinoma tissues and their matched adjacent normal bone and myeloid tissues by Wilcoxon's-paired test, and estimated the differences among the groups in the migration and invasion assays using Student's t-test or one-way ANOVA. A P-value of <0.05 was considered to indicate a statistically significant result.

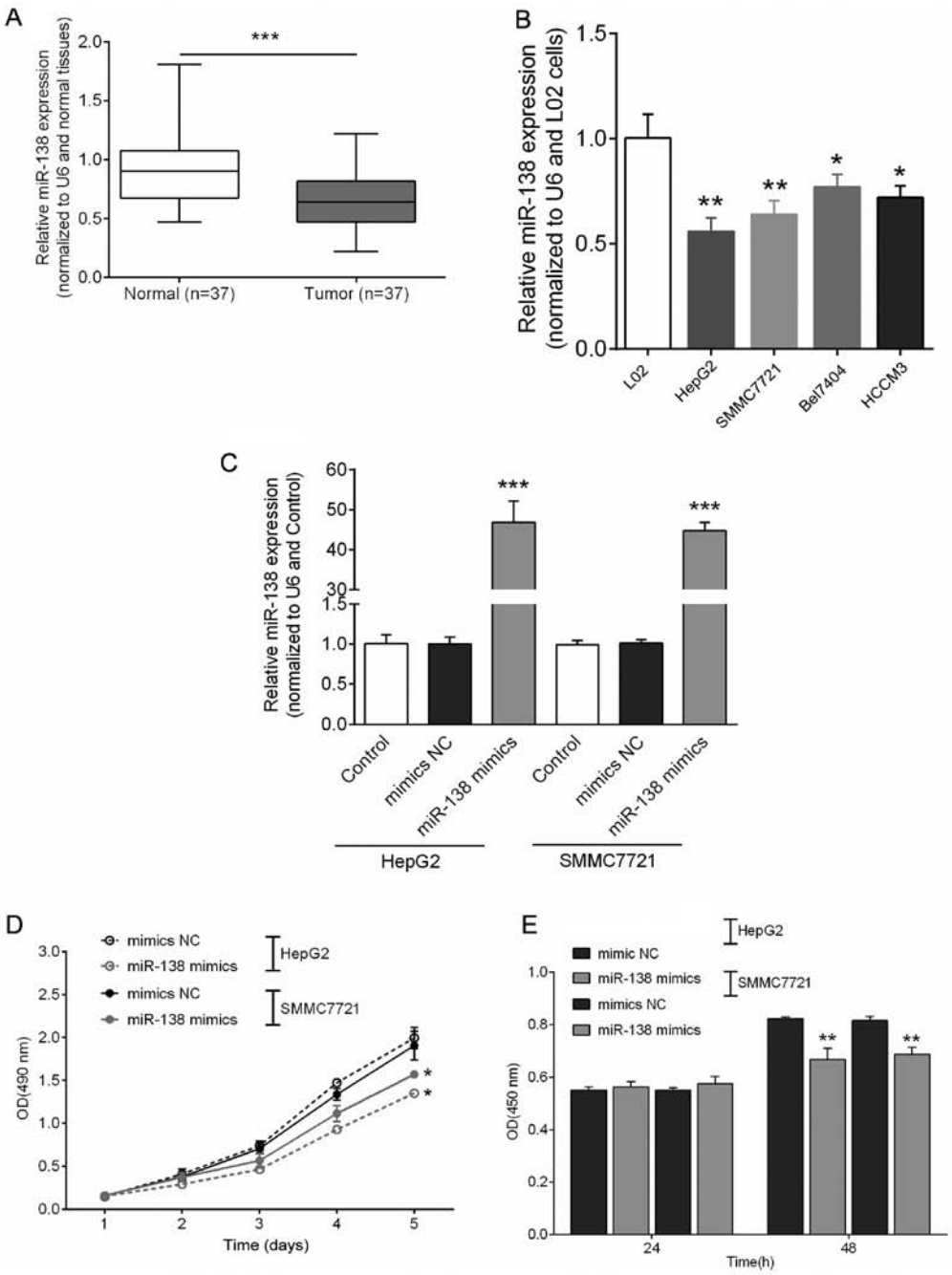

Figure 1. miR-138 is significantly downregulated in hepatocellular carcinoma tissues and cell lines and inhibits the proliferation and invasion of hepatocellular carcinoma cell lines. (A) Among the 37 cases of primary hepatocellular carcinoma tissues and their adjacent normal bone and myeloid tissues, miR-138 expression was at a significant lower level in 27 (73%) hepatocellular carcinoma tissues compared with the adjacent normal tissues. (B) miR-138 expression was attenuated in all of the four hepatocellular carcinoma cell lines compared to that in the L02 cell line. (C) The HepG2 and SMMC7721 cell lines were transfected with miR-138 mimics. The endogenous miR-138 expression in both the HepG2 and SMMC7721 cell lines were induced by miR-138 mimics. (D and E) Cell proliferation was notably inhibited in both HepG2 and SMMC7721 cell lines. The data are presented as the mean ± SD of three independent experiments; *P<0.05, **P<0.01. ***P<0.005.

Results

miR-138 is significantly downregulated in hepatocellular carcinoma tissues and cell lines, and inhibits the proliferation and invasion of hepatocellular carcinoma cell lines. The expression levels of miR-138 in hepatocellular carcinoma tissues and cell lines were detected by performing SYBR-Green quantitative PCR analysis. Among the 37 cases of primary hepatocellular carcinoma and their adjacent normal bone and myeloid tissues, the results revealed that miR-138 expression was at a significant lower expression level in 27 (73%) hepatocellular carcinoma tissues compared with the adjacent normal tissues (Fig. 1A). Moreover, miR-138 expression was attenuated in all of the four hepatocellular carcinoma cell lines compared to that in the L02 cell line (Fig. 1B). The HepG2 and SMMC7721 cell lines were transfected with miR-138 mimics, and the expression of miR-138 was analyzed by real-time PCR. The endogenous miR-138 expression in both HepG2 and SMMC7721 cell lines was induced by miR-138 mimics (Fig. 1C). The effects of miR-138 on cell

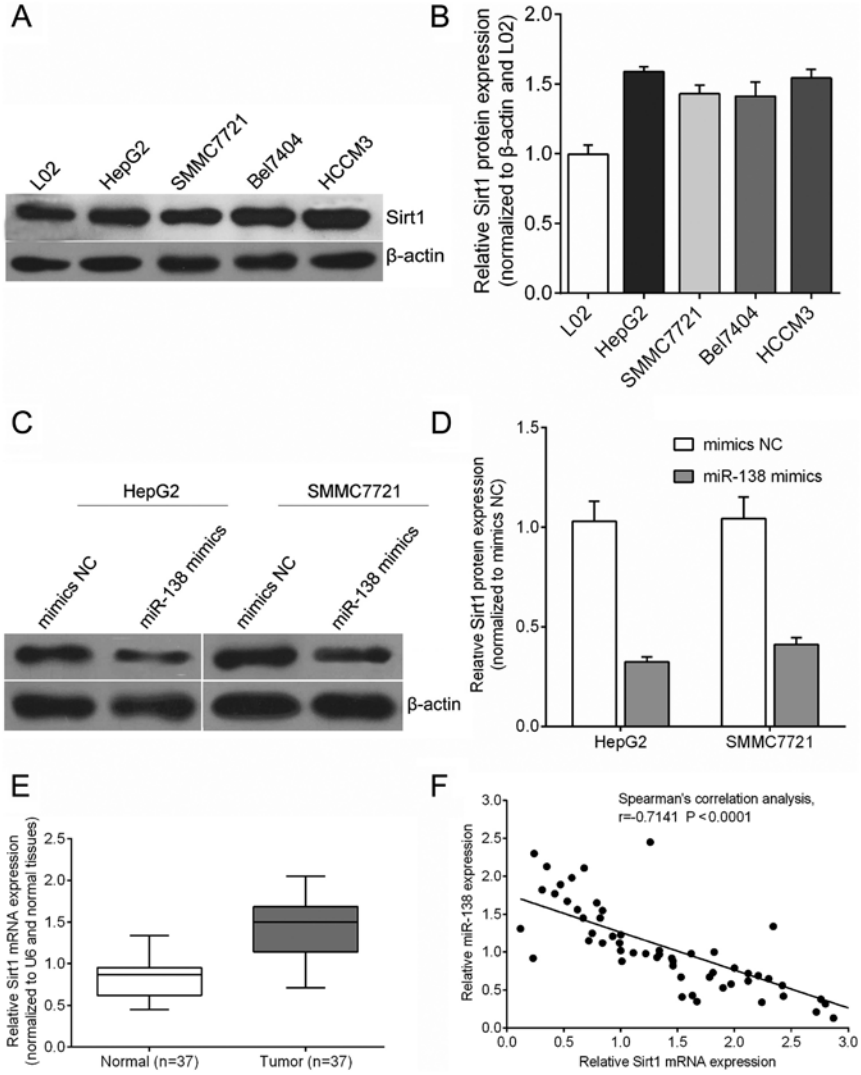

Figure 2. miR-138 efficiently suppresses Sirt1. (A and B) In hepatocellular carcinoma cells including HepG2, SMMC7721, Bel7404 and HCCM3, the Sirt1 protein expression exhibited a higher expression level compared with that in the human normal hepatic cell line L02. (C and D) Quantitative RT-PCR revealed that, at 72 h after infection, the expression of the Sirt1 protein was downregulated in the miR-138 mimic infected cells as compared with miR-SCR (mimics NC) infected cells. (E) The Sirt1 mRNA expression was upregulated in tumor tissues. (F) The miR-138 expression was significantly inversely correlated with Sirt1 mRNA expression levels according to Spearman's correlation test in hepatocellular carcinoma tissues, R^2=0.518 (P<0.0001), confirming that decreased miR-138 expression had a significant association with increased Sirt1 mRNA expression in the same set of hepatocellular carcinoma tissues. The data are presented as the mean ± SD of three independent experiments. Sirt1, sirtuin type 1.

proliferation were determined using CCK-8 assay and BrdU assay, respectively (P<0.05, P<0.01). The results revealed that cell proliferation was notably inhibited in both the HepG2 and SMMC7721 cell lines (Fig. 1D and E).

miR-138 efficiently suppresses Sirt1. In hepatocellular carcinoma cells including HepG2, SMMC7721, Bel7404 and HCCM3, the Sirt1 protein was at a higher expression level compared with that in the human normal hepatic cell line L02 (Fig. 2A and B). To further explore the biological effect of miR-138 in hepatocellular carcinoma tumor progression, we infected a lentivirus carrying miR-SCR or miR-138 mimics into HepG2 and SMMC7721 cells. Quantitative RT-PCR revealed that, at 72 h after infection, the expression of the Sirt1 protein was downregulated in the miR-138 mimic infected cells as compared with the miR-SCR (mimics NC) infected cells (Fig. 2C and D). Moreover, Sirt1 mRNA expression was upregulated in tumor tissues (Fig. 2E). As shown in Fig. 2F, the miR-138 expression was significantly inversely correlated with Sirt1 mRNA expression levels according to Spearman's correlation test in hepatocellular carcinoma tissues, R^2=0.518 (P<0.0001), confirming that decreased miR-138 expression had a significant association with increased Sirt1 mRNA expression in the same set of hepatocellular carcinoma tissues (Fig. 2F).

miR-138 directly targets Sirt1. We used TargetScan (www.targetsan.org) and microRNA.org (www.microRNA.org) to predict the combining sites of the Sirt1 3'UTR with miR-138, and its position was 403-409 of the Sirt1 3'UTR. Then, we performed a sequential substitution of a five-base pair region to produce a mutant vector. The 3'UTR of Sirt1 (wt-Sirt1) was downstream cloned to a luciferase reporter gene to further explore whether the forecasted binding site of miR-138 to the 3'UTR of Sirt1 responded to this modulation. Its mutant version (mut-Sirt1) by mutation at the binding site

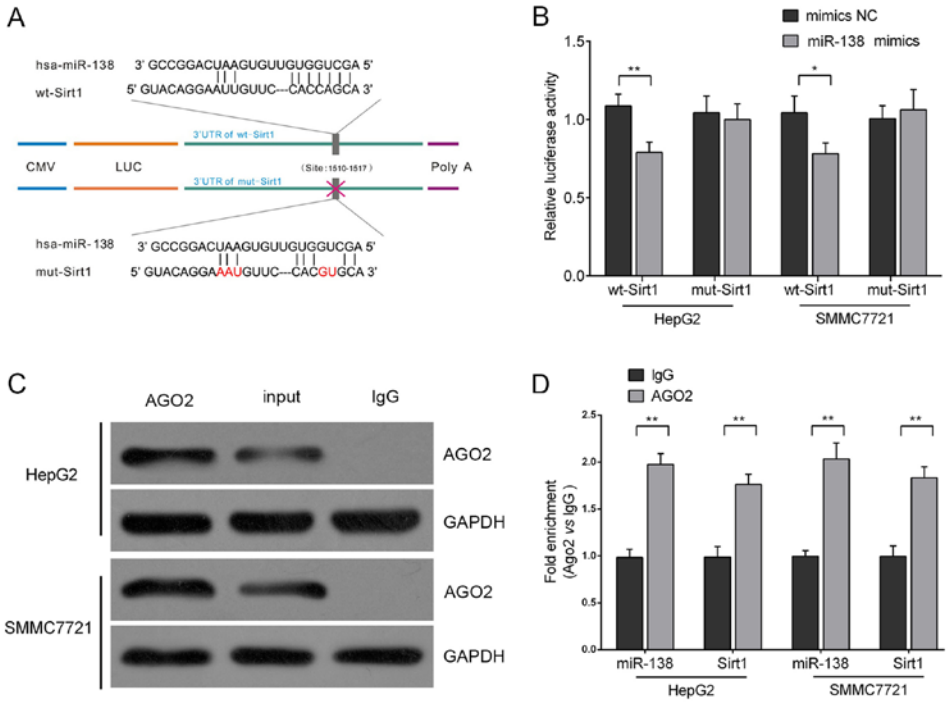

Figure 3. miR-138 directly targets Sirt1. (A) TargetScan (www.targetsan.org) and microRNA.org (www.microRNA.org) were used to predict the combining sites of the Sirt1 3'UTR with miR-138, and its position was 403-409 of the Sirt1 3'UTR. Then, we performed a sequential substitution of a five base pair region to produce a mutant vector. The 3'UTR of Sirt1 (wt-Sirt1) was cloned downstream to a luciferase reporter gene to further explore whether the forecasted binding site of miR-138 to 3'UTR of Sirt1 responded to this modulation. Its mutant version (mut-Sirt1) by mutation at the binding site was also constructed. (B) The wt-Sirt1 vector and miR-138 mimics or scrambled control were co-transfected into HepG2 and SMMC7721 cells. The results revealed that the luciferase activity of the miR-138 mimic transfected cells was markedly attenuated compared to the scrambled (mimics NC) control cells. The mutant putative binding site abolished the suppression of the luciferase activity mediated by miR-138. (C and D) Association of miR-138 and Sirt1 with AGO2. HepG2 and SMMC7721 cellular lysates were used for RNA immunoprecipitation with the AGO2 antibody. The detection of AGO2 and IgG using western blotting (left), and the detection of miR-138 or Sirt1 using qRT-PCR (right). All data of Sirt1 expression were normalized to β-actin mRNA expression levels. The miR-138 expression data were normalized to U6 small RNA expression. The data are presented as the mean ± SD of three independent experiments; *P<0.05, **P<0.01. Sirt1, sirtuin type 1.

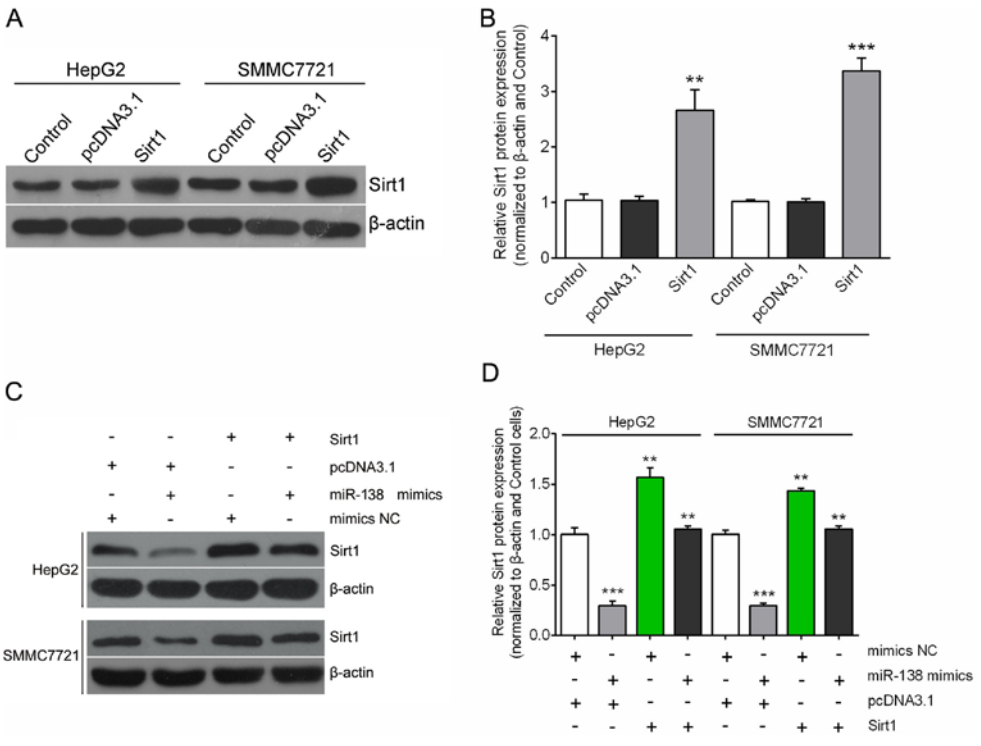

Figure 4. miR-138 overexpression decreases the protein level of Sirt1. (A and B) The expression of Sirt1 was effectively upregulated by the Sirt1 ORF clone in both HepG2 and SMMC7721 cell lines. Sirt1 ORF-expressing plasmid or pcDNA3.1 was transfected into cells treated with miR-138 mimics or mimics NC. (C and D) The forced Sirt1 expression restored significant suppression on Sirt1 expression by miR-138. The data are presented as the mean ± SD of three independent experiments; **P<0.01, ***P<0.005. Sirt1, sirtuin type 1.

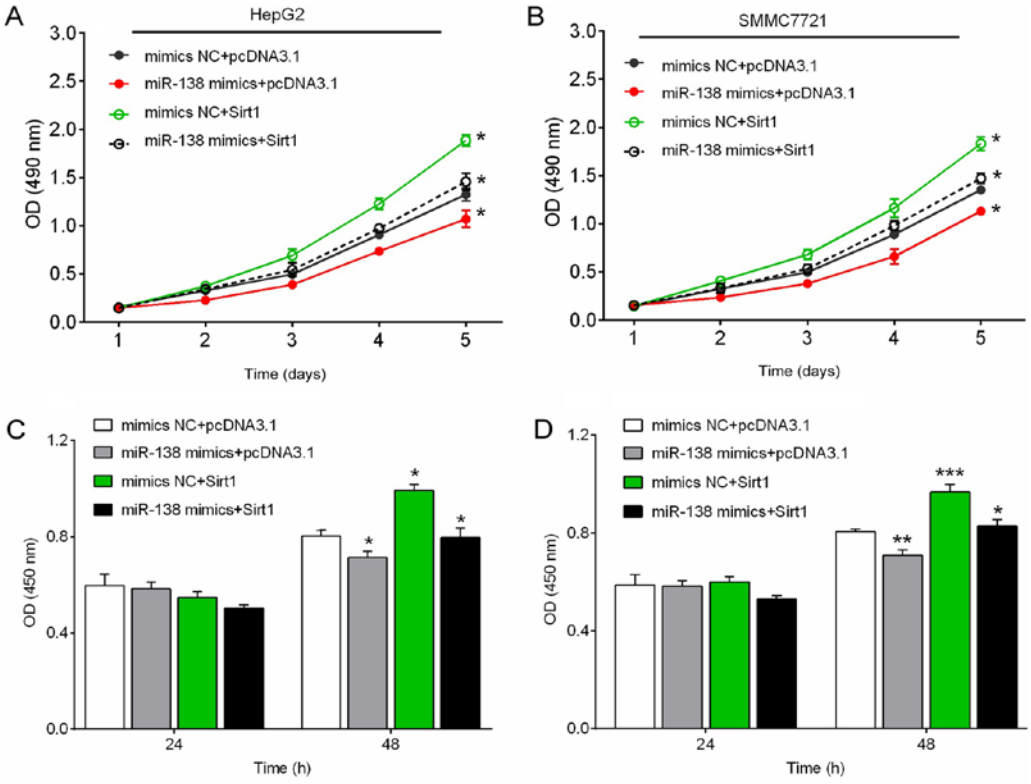

Figure 5. Forced expression of Sirt1 increases hepatocellular carcinoma cell growth. (A and B) The cell viability was determined using CCK-8 assays in response to co-transfection of miR-138 mimics and Sirt1. (C and D) The cell proliferation was determined using BrdU assays in response to co-transfection of miR-138 mimics and Sirt1. The data are presented as the mean ± SD of three independent experiments; *P<0.05, **P<0.01, ***P<0.005. Sirt1, sirtuin type 1.

was also constructed (Fig. 3A). The wt-Sirt1 vector and the miR-138 mimics or scrambled control were co-transfected into HepG2 and SMMC7721 cells. The results revealed that the luciferase activity of the miR-138 mimic transfected cells were markedly attenuated compared to the scrambled control cells (mimics NC). In addition, the mutant putative binding site abolished the suppression of the luciferase activity mediated by miR-138 (Fig. 3B). These data revealed the possible existence of an RNA-induced silencing complex (RISC complex) in both miR-138 and Sirt1. Argonaute2 (AGO2) promotes the target mRNA degradation or inhibits its protein translation; it has been regarded as the core component of RISC (20). In the present study, we further investigated the interaction between Sirt1 and miR-138 in HepG2 and SMMC7721 cells using RNA immunoprecipitation assays with the AGO2 antibody. As exhibited using western blot assays, the AGO2 protein could be precipitated from the cellular extract (Fig. 3C). In RNA extracted from the precipitated AGO2 protein, we detected both miR-138 and Sirt1 with a >1.8~2-fold enrichment compared to IgG (Fig. 3D), indicating that RISC existed in both miR-138 and Sirt1.

miR-138 overexpression decreases the protein level of Sirt1. To further confirm the potential relationship between miR-138 and its downstream gene Sirt1, we assessed the cell growth and motility with overexpression of Sirt1. The expression of Sirt1 was effectively upregulated by the Sirt1-ORF clone in both HepG2 and SMMC7721 cell lines (Fig. 4A and B). We transfected Sirt1 ORF-expressing plasmid or pcDNA3.1 into cells treated with miR-138 mimics or mimics NC. As shown in Fig. 4C and D, the forced Sirt1 expression restored significant suppression on Sirt1 expression by miR-138 (Fig. 4C and D).

Forced expression of Sirt1 increases hepatocellular carcinoma cell growth. Next, we investigated the effect of miR-138/Sirt1 on the viability and proliferation of hepatocellular carcinoma cell using CCK-8 and BrdU assays. The results revealed that, miR-138 mimic transfection significantly suppressed the cell viability and proliferation of hepatocellular carcinoma cells, while forced Sirt1 expression promoted the cell viability and proliferation of hepatocellular carcinoma cells. The suppressive effect of miR-138 on the cell viability and proliferation of hepatocellular carcinoma cells could be partially abolished by forced Sirt1 expression (Fig. 5A-D).

Discussion

Complications arising from metastasis cause most cancer-related deaths. In view of this, treatment for metastatic disease is a vital approach to defeat cancer. Previous studies on tumor invasion and metastasis determined the key role of miRNAs in these processes via the mechanism by which miRNA could regulate various genes which are pivotal to proliferation, invasion or metastasis (21,22). Recently, some miRNAs have been confirmed to have a promotive (23-25) or suppressive (26-28) effect on tumor invasion or metastasis, and provide potential therapeutic targets to defeat metastasis.

In hepatocellular carcinoma, miR-199a-3p expression played a significant role in hepatocellular carcinoma cell growth *in vitro*. Overexpression of miR-199a-3p by

transfection significantly attenuated hepatocellular carcinoma cell growth and migration (29). Moreover, miR-199a-3p was also demonstrated to regulate mTOR and Met to influence doxorubicin sensitivity in liver cancer cells (30). In the present study, we proposed the hypothesis that miR-138 may contribute to the hepatocellular carcinoma metastatic process. Moreover, we confirmed the relationship between miR-138 and Sirt1, which has been identified as a positive tumor metastasis-related gene, and found that miR-138 inhibited hepatocellular carcinoma cell invasion and migration by directly targeting Sirt1. Quantitative RT-PCR results ascertained that miR-138 expression was commonly suppressed in hepatocellular carcinoma cell lines and in 27 out of 37 (73.0%) enrolled hepatocellular carcinoma patients, consistent to previous studies. Subsequently, we restored the expression of miR-138 in HepG2 and SMMC7721 cells and found that miR-138 suppressed cell proliferation and invasion. Collectively, it was determined that miR-138 contributed to the processes of metastasis. Furthermore, the expression levels of miR-138 had a reverse correlation with Sirt1 mRNA levels in hepatocellular carcinoma tissues. Sirt1 has been identified as an independent prognostic indicator of metastasis formation and metastasis-free survival. The present study, ascertained a crucial molecular relationship between miR-138 and Sirt1. We revealed that, upregulation of miR-138 expression in HepG2 and SMMC7721 cells effectively downregulated Sirt1 expression at both the mRNA and protein levels, while forced expression of Sirt1 reversed the expression of Sirt1. A potential inverse regulatory trend of miR-138 and Sirt1 was noted in hepatocellular carcinoma cells, and the main effect of Sirt1 on the cells was an autocrine effect, due to the downregulation of the level at the cellular Sirt1 mRNA and protein by miR-138.

Furthermore, by performing a luciferase reporter assay we confirmed that miR-138 directly targets the Sirt1 gene through binding to the unique complementary site within its 3'UTR. This result ascertained the key role of miR-138 in cellular proliferation, migration and invasion via direct inhibition of the expression of Sirt1. The aforementioned results confirmed the inhibitory effect of miR-138 on Sirt1, and in addition in part elucidated a potential molecular mechanism by which miR-138 participated in hepatocellular carcinoma invasion. Recently, Hurst *et al* (31) suggested a new series of cancer-related miRNAs known as metasta-miRs that are observably involved in the metastatic processes. For example, miR-21 is an inducer of metastasis that promotes cell survival, migration, invasion and metastasis (32-34), while the miR-200 family plays an essential role in tumor suppresion and its deficiency contributes to the EMT phenotype (35-37). miR-204, whose expression was downregulated in different cancer cell lines, has currently been identified as a direct post-transcriptional suppressor of Snai1 mRNA and consistent with its predicted tumor-suppressive role. Suppressed expression of miR-204 led to loss of adhesion between cells supporting the EMT-related properties of Snai1 (38). These metasta-miRs represent potential candidate cancer prognostic markers and therapeutic targets for metastatic cancers. In the present study, we revealed that miR-138 functioned as a metasta-miR via targeting of Sirt1. Moreover, miR-138 could potentially be a significant diagnostic and prognostic tool to hepatocellular carcinoma.

In conclusion, we newly described the link between miR-138 and Sirt1 and elucidated a potential mechanism in which Sirt1 is regulated by miR-138 and contributes to the inhibition of hepatocellular carcinoma cell proliferation and invasion. Moreover, restoration of miR-138 expression was markedly implicated in the clinical management of hepatocellular carcinoma.

Acknowledgements

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