MicroRNA-199a-5p inhibits tumor proliferation in melanoma by mediating HIF-1α

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Abstract. The expression of hypoxia-inducible factor 1α (HIF-1α) is often abundant in human cancer and it is associated with poor prognosis. The present study aimed to investigate its regulation by microRNA (miRNA). The expression of miRNA-199a-5p (miR-199a-5p) in melanoma was detected by quantitative polymerase chain reaction on samples from 25 melanoma patients. The target of miR-199a-5p was predicted and demonstrated by a dual-luciferase reporter system. The effects of miR-199a-5p on melanoma cells were assayed in B16 and HME1 melanoma cell lines. Furthermore, the potential of miR-199a-5p as a therapeutic target was illustrated in xenograft nude mice models. Low expression of miR-199a-5p in tumor melanoma tissue samples from patients was associated with high histological grade and advanced tumor stage. The 3'-untranslated region of HIF-1α was identified as a target of miR-199a-5p by Targetscan software. The dual-luciferase reporter assay demonstrated that miR-199a-5p transfection of mimics decreased the luciferase activity significantly ($P<0.05$). In the B16 and HME1 cell lines, overexpression of miR-199a-5p suppressed cell proliferation and arrested the cell cycle in the G_1 phase. *In vivo* overexpression of miR-199a-5p significantly suppressed xenograft growth and downregulated the expression of HIF-1α ($P<0.05$). The results from the present study suggest that miR-199a-5p suppressed melanoma proliferation via HIF-1α, suggesting it may be a potential therapeutic target for melanoma treatment.

Introduction

Melanoma is the most fatal type of skin cancer (1); however, when it is diagnosed at an early stage, there is a relatively high success rate of treatment decreasing tumor size (2). However, melanoma diagnosed at an advanced stage has been associated with poor prognosis. The predominant therapeutic strategies for melanoma are chemo-and immunotherapy, or radiation therapy (3), however, these strategies also affect non-cancerous cells and result in side effects (4). Thus, elucidation of the molecular mechanisms underlying melanoma initiation and progression is required to develop effective therapeutic strategies against tumorigenesis.

Hypoxia-inducible factor 1α (HIF-1α) activates the transcription of genes that are involved in cancer initiation and progression and associated with cell survival and cell invasion (5-7). Overexpression of HIF-1α has been associated with poor prognosis and high mortality rate in a number of cancer types, including breast cancer (7-9). In clinical applications, HIF-1α may be useful as a marker to indicate tumor growth (10). It has been reported that a decrease in HIF-1α reduces the capacity to release vascular endothelial growth factor in hypoxia and resulted in inhibition of solid tumor growth (11). Thus, inhibitors of HIF-1α may be potential anticancer therapeutic agents.

MicroRNAs (miRNAs) are non-coding RNAs (length, ~22 nt) (12), they target transcripts, particularly the 3'-untranslated region (UTR) to regulate gene expression (13). miRNAs have been indicated to be involved in a similar function in other organisms (14-16). Current research into miRNA-based anticancer therapeutic strategies aims to regulate and modulate various cancer chemoprophylaxis agents (17). Thus, the development of a specific and efficient target for miRNA molecules is key. Based on previous studies (10,11), HIF-1α may be a potential target for miRNA-based anticancer therapeutic strategies.

The present study focused on the expression profile of miR-199a-5p in melanoma. The expression of miR-199a-5p and its association with poor prognosis were detected and the suppressive effects of miR-199a-5p on melanoma cells were demonstrated. The downregulation of HIF-1α by miR-199a-5p was also demonstrated in melanoma cells and the effect of miR-199a-5p on cell viability and apoptosis was investigated. miR-199a-5p was demonstrated to suppress melanoma proliferation in a nude mouse model. The present results suggest that miR-199a-5p inhibited melanoma via targeting of HIF-1α.

Materials and methods

Clinical samples. The clinical samples (cancer tissues and adjacent tissues) from 25 patients, including 13 men and

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12 women, were collected from Xiangya Hospital, Central South University (Changsha, China). The experiments were approved by the Xiangya School of Medicine Research Ethics Committee (Changsha, China). Informed consent was obtained according to the Declaration of Helsinki. The 25 patients enrolled in the present study had not undergone surgical removal of the lesion or any prior treatment. Clinical characteristics of the patients is presented in Table I. The control samples were adjacent non-cancerous tissue samples obtained from the same patients.

Cell culture and transfection. B16 and HME1 melanoma cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in 5% CO_2 at 37˚C.

To understand the effect of miR-199a-5p on melanoma cells, the cells were divided into groups: i) Control, ii) mimics (100 nmol), and iii) inhibitors (100 nmol) with n=4 in each group. The cells ($1x10^6$ cells/well) were plated into 6-well plates and incubated at 37˚C for 24 h in 5% CO_2. The cells were transfected using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. The mimics and inhibitors were purchased from Sangon Biotech Co., Ltd. (Shanghai, China).

The RNA interference was performed as described previously (18). Briefly, 100 nmol HIF-1α siRNA (sense, 5'-CUG AUGACCAGCAACUUGA-3' and antisense, 5'-UCAAGU UGCUGGUCAUCAG-3'), or 100 nmol miR-199a-5p mimics, were transfected into B16 cells using Lipofectamine 2000. Following 24 h, the cells were analyzed by flow cytometry, MTT and colony formation assay.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNAs were isolated using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol, and were treated with DNase I (Sangon Biotech Co., Ltd.) to remove contaminating genomic DNA. The purity of the extracted RNA was assessed by measuring the optical density ratio (A260/A280) using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc.). The expression level of miR-199a-5p was determined using the TaqMan® microRNA Assays kit (cat. no. 4324020; Applied Biosystems; Thermo Fisher Scientific, Inc.), in which U6 served as an endogenous control. In order to determine the expression level of HIF-1α, total RNA (1 μg) was reverse transcribed into cDNA using the TaqMan® Reverse Transcription kit (cat. no. N8080234; Applied Biosystems; Thermo Fisher Scientific, Inc.). qPCR was performed using the TaqMan® Universal PCR Master Mix (cat. no. 4324018; Applied Biosystems; Thermo Fisher Scientific, Inc.) on the ABI 7500 thermal cycler (Applied Biosystems; Thermo Fisher Scientific, Inc.). The primers were as follows: Sense, 5'-CCACAGGAC AGTACAGGATG-3' and antisense, 5'-TCAAGTCGTGCT GAATAATACC-3' for HIF-1α and sense, 5'-AGGGCTGCT TTTAACTCTGGT-3' and antisense, 5'-CCCCACTTGATT TTGGAGGGA-3' for GAPDH (Sangon Biotech Co., Ltd.). GAPDH served as an endogenous control. The cycling conditions were as follows: 95˚C for 10 min, followed by 40 cycles at 95˚C for 15 sec and 60˚C for 1 min. The relative mRNA expression was calculated from three independent experiments using the $2^{-\Delta\Delta Cq}$ method (19). Melting curve analyses were performed to assess whether the amplifications were specific.

Table I. Characteristics of patients.

Variable	Data
Age (years)	
Mean	45
Median	47.62
Range	37-69
Histological grade	
I	10
II	6
III	9
Tumor stage	
I	7
II	8
III	5
IV	5

Western blot. The tested cells were lysed using radioimmunoprecipitation buffer (Beyotime Institute of Biotechnology, Haimen, China) and the quantity of protein was detected using a BCA kit (Beyotime Institute of Biotechnology). For each sample, 15 μg total protein was separated on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinyl difluoride membranes (EMD Millipore, Billerica, MA, USA). Following blocking with 4% non-fat milk, the membranes were incubated with rabbit anti-HIF-1α (1:1,000; cat. no. SAB2104366) and anti-GAPDH (1:1,000; cat. no. SAB2100894) polyclonal antibodies (Sigma-Aldrich), followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) secondary antibody (1:2,000; cat. no. A0545) and an enhanced chemiluminescent substrate (ECL Plus, GE Healthcare Life Sciences, Chalfont, UK). The protein bands were visualized using X-ray paper (Kodak, Rochester, NY, USA)

MTT assay. The cells were plated into 96-well plates at concentration of $5x10^3$ cells/well. Following culture for 24 h, the cells were transfected with 100 nmol miR-199a-5p mimics or 100 nmol miR-199a-5p inhibitors (n=3 for each group). Following transfection, the cells were treated using MTT solution (Sigma-Aldrich) according to the manufacturer's protocols. The absorbance at a wavelength of 570 nm was measured on a DU 800 UV/Vis spectrophotometer (Beckman Coulter, Inc., Brea, CA, USA).

Colony formation assay. For the colony formation assay, 1,000 cells/well were plated into the 6-well plates and incubated for 7 days. Following removal of the culture medium and fixing in 30% formaldehyde (Sangon Biotech Co., Ltd.) for 15 min, the cells were stained using 0.2% crystal violet (Sangon Biotech Co., Ltd.). The colonies from three independent experiments

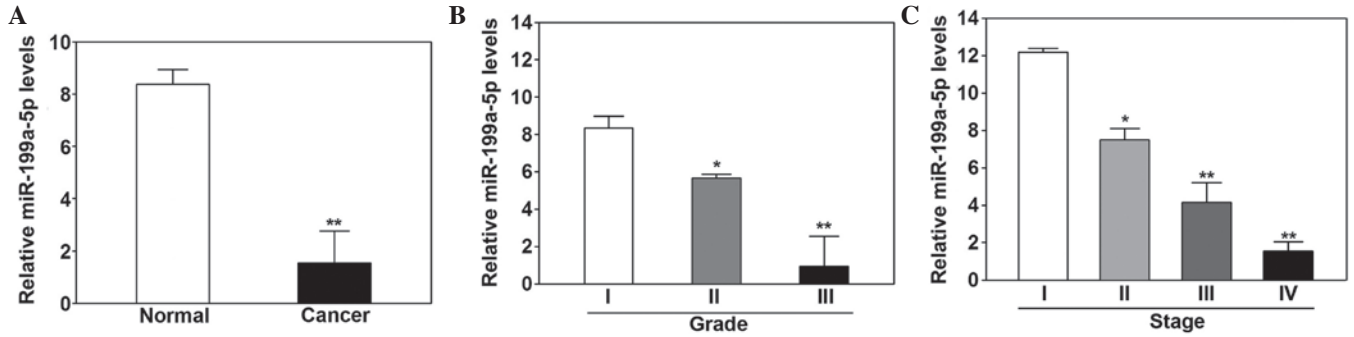

Figure 1. Expression of miR-199a-5p was downregulated in melanoma tissue samples. (A) Compared with the normal control, the expression of miR-199a-5p was significantly lower in the melanoma tissue samples. *P<0.05, **P<0.01 vs. the normal control group. (B) A low level of miR-199a-5p was positively correlated with histological grade. *P<0.05, **P<0.01 vs. Grade I. (C) A high level of miR-199a-5p was correlated with advanced tumor stage. *P<0.05, **P<0.01 vs. Stage I. miR-199a-5p, microRNA-199a-5p.

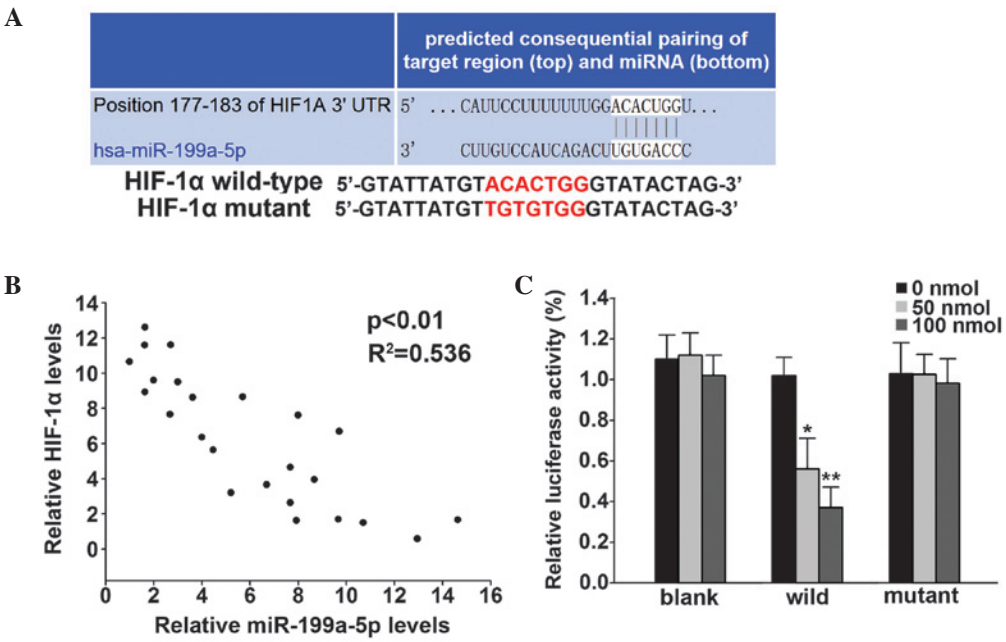

Figure 2. miR-199a-5p directly targeted HIF-1α. (A) The Targetscan software predicted a miR-199a-5p binding site within the 3'-UTR of HIF-1α (upper section. A wild-type and a mutant 3'-UTR of HIF-1α were constructed to determine the interaction between miR-199a-5p and 3'-UTR of HIF-1α (lower section). (B) Correlation of mRNA expression level of miR-199a-5p and the expression level of HIF-1α in the melanoma tissue samples. (C) In B16 cells, 50 nmol and 100 nmol miR-199a-5p mimics suppressed the luciferase activities in the wild-type group. *P<0.05, **P<0.01 vs. the control group. miR-199a-5p, microRNA-199a-5p; HIF-1α, hypoxia-inducible factor 1α; UTR, untranslated region.

were observed under an optical microscope (Leica DMI6000B; Leica Microsystems GmbH, Wetzlar, Germany).

Flow cytometry. Cells ($1x10^4$) from all groups were fixed in 90% methanol for 2 h and resuspended in phosphate-buffered saline with 0.1% bovine serum albumin, 0.05% Triton X-100 and 50 μg/ml RNase A (Sigma-Aldrich). Annexin V-fluorescein isothiocyanate and propidium iodide (BioVision, Inc., Milpitas, CA, USA) staining was performed according to the manufacturer's protocols. The cell counting was performed on a BD FACSCalibur™ system analyzed using BD FACSDiva Software 6.0 (BD Biosciences, Franklin Lakes, NJ, USA). Triplicate biological repeats were measured.

Luciferase assay. Wild-type and mutant 3'-UTR of HIF-1α containing the target sequence were synthesized by Sangon Biotech Co., Ltd. and cloned into the pMIR-Report vector (Applied Biosystems; Thermo Fisher Scientific, Inc.). The B16 cells were transfected with mimics (50 and 100 nmol) and then co-transfected with wild-type and mutant 3'-UTR of HIF-1α by Lipofectamine 2000. After 48 h, the cells were lysed using FastBreak™ Cell Lysis Reagent (Promega Corporation, Madison, WI, USA) and the cell lysates were incubated with dual-luciferase reagents (Promega Corporation) and the luciferase activities were assayed by Dual-Luciferase Reporter assay system (Promega Corporation). The experiments were repeated three times.

In vivo xenograft assay. A total of 12 female nu/nu mice (age, 6 weeks; weight, 20.03±1.92 g) were obtained from the Xiangya School of Medicine Research. The mice were maintained at 23˚C under a 12-h light/dark cycle with

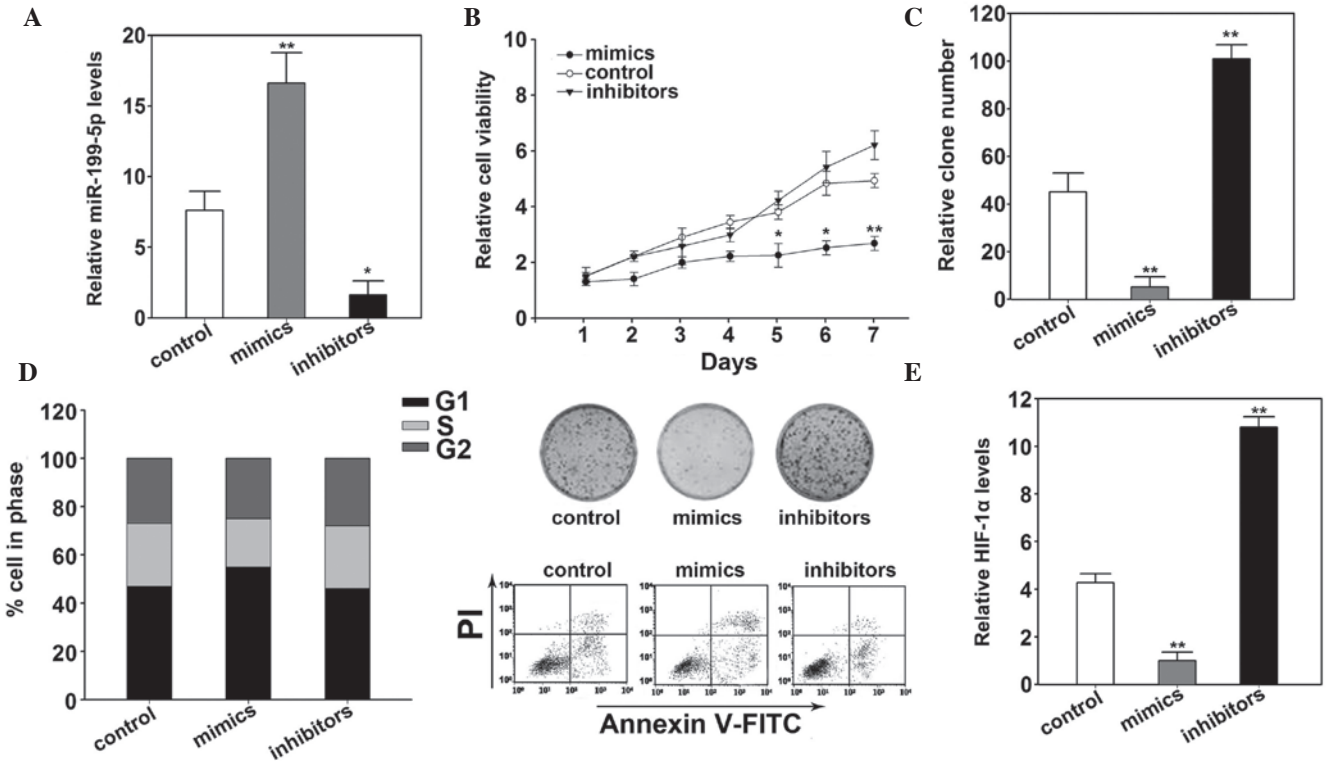

Figure 3. miR-199a-5p depressed cellular proliferation and arrested cell cycle progression via inhibiting HIF-1α in B16 cells. (A) The expression levels of miR-199a-5p were markedly higher and lower in the mimics and inhibitors groups, respectively. (B) Compared with the control, miR-199a-5p mimics accelerated the cellular growth of B16 cells. (C) The colony formation was decreased and stimulated by miR-199a-5p mimics and inhibitors, respectively. (D) miR-199a-5p mimics arrested the cell cycle in the G1 phase, as analyzed by flow cytometry. (E) Depression and stimulation of HIF-α expression levels by miR-199a-5p mimics and inhibitors, respectively. *P<0.05, **P<0.01 vs. the control group. miR-199a-5p, microRNA-199a-5p; HIF-1α, hypoxia-inducible factor 1α; FITC, fluorescein isothiocyanate.

ad libitum access to food and water. The mice were injected with $1x10^7$ B16 cells (with or without miR-199a-5p transfection of mimics) into to the posterior flank. The volume [volume = (length x width^2)/2] and weight of the tumors were measured once a week. After 4 weeks, the mice were sacrificed by cervical dislocation following anesthetization with 10% chloral hydrate (4 ml/kg via intraperitoneal injection; Sigma-Aldrich) and the xenograft tissues were collected for immunohistochemical staining.

Immunohistochemical staining assay. The xenograft tissue samples were harvested from all mice and then fixed in 10% formalin and paraffin-embedded (both Sangon Biotech Co., Ltd.), prior to cutting into 6-μm sections. Following deparaffinzation using xylene (Sangon Biotech Co., Ltd.), the tissues were incubated with rabbit anti-HIF-1α and anti-GAPDH polyclonal antibodies (1:200), followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:1,000) and and diaminobenzidine (Beyotime Institute of Biotechnology). Subsequently, the tissue sections were visualized using the Leica DMI6000B microscope. The experiments were repeated three times.

Statistical analysis. All the values are presented as the mean ± standard deviation. Statistical analyses were performed using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance was used to determine differences among groups. P≤0.05 was considered to indicate a statistically significant difference. If the *F* ratios exceeded the critical value (P≤0.05), the Newman-Keul's post-hoc test was performed to compare the groups.

Results

Decreased expression of miR-199a-5p was demonstrated in melanoma tissue samples. The expression levels of miR-199a-5p were significantly lower in melanoma tissue samples compared with the adjacent normal tissue samples (P<0.01, Fig. 1A). Low expression levels of miR-199a-5p were associated with higher histological grade (P=0.031; Fig. 1B), and more advanced tumor stage (P=0.042; Fig. 1C).

miR-199a-5p directly targeted the 3'-UTR of HIF-1α. Targetscan software (www.targetscan.org) was used to predict the target of miR-199a-5p; it was identified as the 3'-UTR of HIF-1α (Fig. 2A). The association between miR-199a-5p and HIF-1α was analyzed in patients. The mRNA expression level of HIF-1α was negatively correlated with miR-199a-5p (P<0.001, R^2=0.536; Fig. 2B). Using the dual-luciferase reporter system, the direct regulation of miR-199a-5p on 3'-UTR of HIF-1α was confirmed. The 50 and 100 nmol miR-199a-5p transfection of mimics significantly decreased the luciferase activities by 44% (P<0.05) and 73% (P<0.01), respectively, in the wild-type group (Fig. 2C) while no marked difference was detected in the mutant group (Fig. 2C).

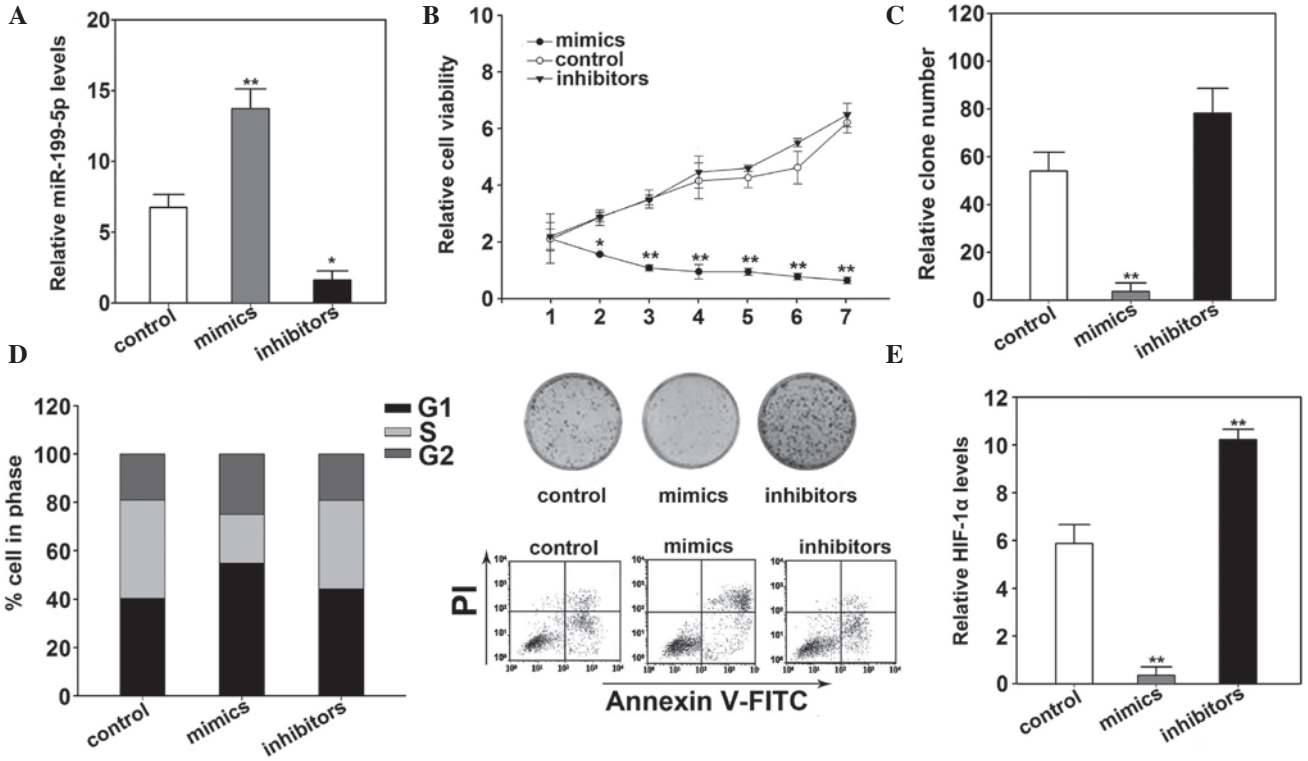

Figure 4. miR-199a-5p reduced cellular proliferation and arrested cell cycle progression via inhibiting HIF-1α in HME1 cells. (A) The expression of miR-199a-5p was markedly higher and lower in the mimics and inhibitors group, respectively. (B) Compared with the control, miR-199a-5p mimics markedly increased the cellular growth of HME1 cells. (C) Colony formation was reduced and stimulated by miR-199a-5p mimics and inhibitors, respectively. (D) miR-199a-5p mimics arrested the cell cycle in the G1 phase, as analyzed by flow cytometry. (E) Depression and stimulation of HIF-α by miR-199a-5p mimics and inhibitors, respectively. *P<0.05, **P<0.01. vs. the control group. miR-199a-5p, microRNA-199a-5p; HIF-1α, hypoxia-inducible factor 1α; PI, propidium iodide.

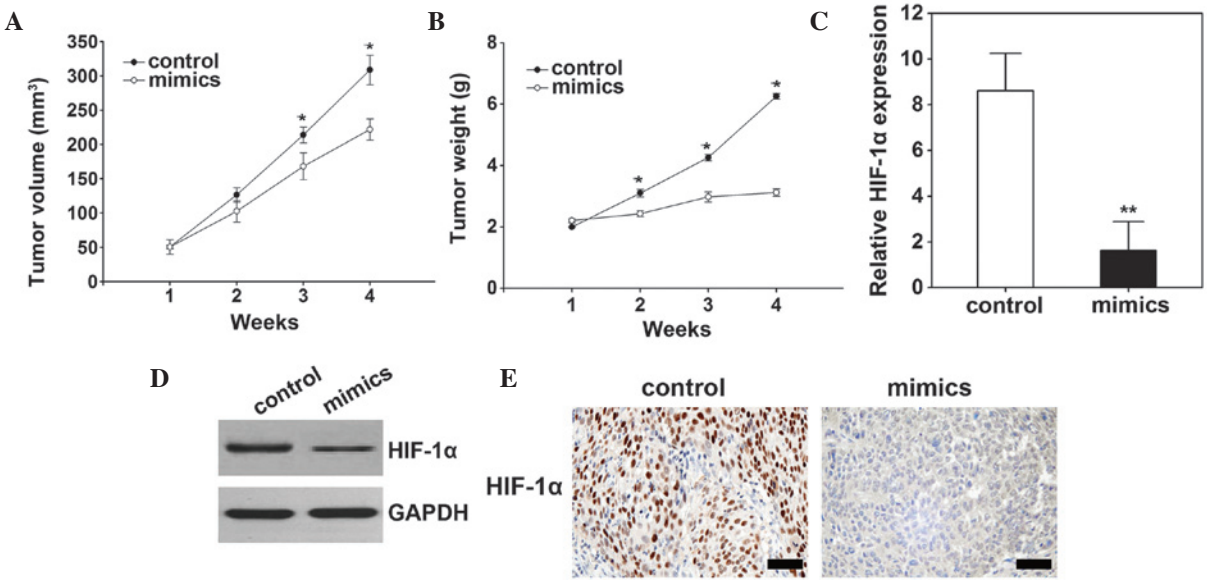

Figure 5. Inhibition of tumor growth by miR-199a-5p mimics. (A and B) Tumor growth and volume indicated that the miR-199a-5p depressive effect on tumor growth *in vivo*. HIF-1α expression was depressed by miR-199a-5p mimics in tumor tissues by (C) reverse transcription-quantitative polymerase chain reaction, (D) western blotting and (E) immunohistochemical staining assay. Data are presented as the mean ± standard deviation. *P<0.05, **P<0.01 vs. the control group.

miR-199a-5p suppressed cell proliferation of B16 cells by targeting HIF-1α. The effects exerted by miR-199a-5p on B16 cells were detected by transfection with miR-199a-5p mimics and inhibitor. The results of the RT-qPCR demonstrated that groups transfected with miR-199a-5p mimics and inhibitors groups showed significantly increased (P<0.01) and decreased (P<0.05) miR-199a-5p expression levels, respectively (Fig. 3A). Furthermore, overexpression of miR-199a-5p inhibited cell viability (P=0.012; Fig. 3B). The colony formation was also decreased by miR-199a-5p overexpression (P=0.031; Fig. 3C). Apoptosis was investigated using flow cytometry, the miR-199a-5p mimics group indicated increased apoptosis

compared with the control (P=0.031; Fig. 3D). Compared with the control groups, miR-199a-5p overexpression arrested the cell cycle into G_1 phase (P=0.011; Fig. 3D). The expression of HIF-1α was decreased following transfection of miR-199a-5p mimics (P=0.014; Fig. 3E).

miR-199a-5p suppressed cell proliferation of HME1 cells via targeting HIF-1α. Similar effects of miR-199a-5p on HME1 were observed. The expression levels of miR-199a-5p increased significantly following transfection of mimics (P<0.05; Fig. 4A). The transfection also significantly inhibited the cell viability (P=0.023; Fig. 4B). Overexpression of miR-199a-5p also decreased the colony formation in HME1 cells (P=0.019; Fig. 4C). Flow cytometry analysis demonstrated increased apoptosis with overexpression of miR-199a-5p (Fig. 4D). The cells were arrested in G_1 phase significantly more than the control cells (P=0.022, Fig. 4D). In addition, miR-199a-5p mimics decreased HIF-1α mRNA and protein expression levels (P=0.032; Fig. 4E).

Inhibition of miR-199a-5p suppressed tumor growth in vivo. The effects of miR-199a-5p on tumor growth were observed in the nude mice model. The results demonstrated that following treatment with miR-199a-5p mimics, the tumor volume (P=0.012; Fig. 5A) and weight (P=0.023; Fig. 5B) significantly increased. Consistently with *in vitro* study, overexpression of miR-199a-5p decreased HIF-1α mRNA and protein expression levels in tumor tissue samples (Fig. 5C-E).

Discussion

A previous study has demonstrated that HIF-1 was significantly increased in malignant tumor samples (20). HIF-1 consists of HIF-1α, oxygen-responsive, and HIF-1β, constitutive, subunits (21). The induced expression of HIF-1 by hypoxia resulted in the proliferation of malignant melanoma cells (22). Furthermore, HIF-1 knockdown suppressed the proliferation of melanoma cells, which suggested that the inhibition of HIF-1 is a potent therapy for melanoma (22). An effective treatment on targeting HIF-1 remains to be developed in other cancer types. The present study demonstrates that HIF-1α was a direct target of miR-199a-5p and that overexpression of miR-199a-5p reduced melanoma proliferation suggesting this may be a potential therapeutic strategy for melanoma.

Research into miRNA as a therapeutic strategy has developed in recent years (23-25). However, only miR-122 as a therapeutic agent for hepatitis C virus has entered phase 2 clinical trials (26). However, the development of miRNAs as critical regulators in human disease has shown rapid progress and may result in a novel class of therapeutic agents. In melanoma, miRNAs may be identified as oncomiRs or tumor suppressors (27). The results from the present study indicated that miR-199a-5p is a tumor suppressor, which suppressed proliferation in the cells via direct targeting of HIF-1α. The expression levels of miR-199a-5p also inhibited the effects of HIF-1α in cancer cells. To the best of our knowledge, the present study is the first to demonstrate the target of miR-199a-5p in malignant melanoma.

Consistent with the results from the present study, miR-199a-5p was demonstrated to be a tumor suppressor in aggressive human cancer cells (28,29). Decreased expression of miR-199a-5p increased cell invasion via downregulating the expression of discoidin domain receptor tyrosine kinase 1 in human hepatocellular carcinoma (29). Furthermore, miR-199a-5p is highly expressed in Brahma-related gene 1 (Brm)-deficient tumor cell lines, however, this is reduced in Brm-expressing tumor cells (28). The luciferase assay demonstrated that miR-199a-5p directly targeted the Brm subunit of SWI/sucrose non-fermentable and generate a double-negative feedback loop in human cancers (28). In another previous study, miR-199a-5p was demonstrated to target HIF-1α and sirtuin 1, during hypoxia preconditioning antagonistically to the AKT and beta-adrenergic signaling pathways in neonatal cardiac myocytes, which may lead to suppression of cancer cell proliferation (30). The results from previous studies and the current study suggest that miR-199a-5p inhibits tumor cell proliferation via targeting multiple genes, further research is required in the future.

The cell cycle controls cell proliferation and tumor aggression during tumor growth. The present study demonstrated that low expression of miR-199a-5p is associated with tumor growth. A previous study indicates that miRNAs may activate their target genes in arrested cells by repressing their targets (16). Thus, miR-199a-5p may affect cell cycle dynamics that induce accumulation of cells at the G_2/M phase in the MDA-MB-231 breast cancer line (31). However, the effect was not observed in the MCF7 breast cancer cell line (31). The present study indicated that overexpression of miR-199a-5p arrested the cell cycle in the G_1 phase, suggesting an underlying molecular mechanism of miR-199a-5p in suppressing proliferation of malignant melanoma.

In conclusion, the present study observed low expression of miR-199a-5p was demonstrated malignant melanoma. Overexpression of miR-199a-5p inhibited melanoma cells *in vitro* by arresting the cells in G_1 phase. Furthermore, HIF-1α was identified as a target of miR-199a-5p and the results from the present study suggest that overexpression of miR-199a-5p may be a potential therapeutic strategy for melanoma.

Acknowledgements

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