MicroRNA-124a inhibits cell proliferation and migration in liver cancer by regulating interleukin-11

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Abstract. Liver cancer is the sixth most common malignant tumour and ranks in the top three cancers with regard to mortality due to metastasis and postsurgical recurrence. It is significant to understand the mechanisms underlying liver cancer for diagnosis and treatment. Cumulative evidence suggests that the abnormal regulation of microRNAs (miRNAs/miRs) may contribute to the development and metastasis of cancer. miR-124a acts as a tumour suppressor in osteosarcoma, endometrial carcinoma, prostate cancer, and glioblastoma. However, the effects of miR-124a in liver cancer and its biological mechanism are not fully understood. It has been demonstrated that miR-124a is downregulated and interleukin (IL)-11 is upregulated in the liver cancer tissues. In the present study, miR-124a upregulation inhibited cell proliferation, migration and promoted cell apoptosis. Through a dual-luciferase reporter assay, it was verified that IL-11 is a direct target of miR-124a. Furthermore, the overexpression of miR-124a repressed the secretion of IL-11 from hepatoma cells. Finally, it was identified that mimics of miR-124a increased the expression of tissue inhibitor of matrix metalloproteinase-2 (TIMP-2) and Caspase-3 and decreased the expression levels of matrix metalloproteinase 2 (MMP2), MMP9, B-cell lymphoma 2, signal transducer and activator of transcription 3 (STAT3), and phosphorylated-STAT3. In conclusion, the results indicated that miR-124a has an important role as a tumour suppressor gene by targeting IL-11. These findings may provide novel insights for clinical treatments to prevent the development of liver cancer.

Introduction

Globally, liver cancer has the sixth highest incidence for cancer, and it ranks in the top three for cancer mortality. In some Asian countries, liver cancer is the leading cause of cancer death in men (1). In developed countries, such as the United States and Europe, the incidence and mortality of liver cancer is also increasing year after year (2,3). Ninety percent of liver cancer-induced deaths are caused by tumour metastasis; bone is a common site of metastasis, and the spine is the most common site of bone metastasis (BM) (4). Liver cancer patients with BM often experience severe pain with decreased quality of life. The prognosis for these patients is poor: The one-year survival rate of patients with spinal metastasis is only 30 percent after surgery (5,6). So, it is of important clinical value to explore the mechanism of liver cancer BM, especially the mechanism of spinal metastasis, and to develop new biomarkers to evaluate the BM risk of primary liver cancer.

Liver cancer is a complex disease involving epigenetic instability, chromosomal instability, and expression abnormalities, including miRNAs. miRNAs are a type of small non-coding RNAs with lengths of approximately 20-24 nt which can bind to the 3'UTR of target genes through complementary base pairing and thereby inhibit the expression of the target genes (7). Over 1,000 miRNAs have been found to play vital roles, and it has been estimated that over 50% mammalian protein coding genes may be regulated by miRNAs (8). By regulating the expression level of different target genes, miRNAs are widely involved in the regulation of various physiological processes, including organ development, cell differentiation, and immune response, and are closely related to tumour development (9). Some miRNAs even act as oncogenes or tumour suppressors and modulate tumour progression and metastasis (10).

Recently, cumulative evidence has suggested that the dysregulation of miRNAs plays a pivotal role in the formation and progression of liver cancer (11). Thus far, several miRNAs are previously reported to be dysregulated in liver cancer, including miR-21, miR-122, miR-181b, miR-338, and miR-491 (12-16), which are all closely associated with the migration and metastasis of liver cancer. miR-124a functions as a tumour suppressor in osteosarcoma, endometrial carcinoma, prostate cancer, and glioblastoma (17-20). It is also downregulated in liver cancer tissues (21). However, the role of miR-124a in liver cancer and potential targets of miR-124a in hepatoma cells have not been studied previously. In this study, we identified that interleukin (IL)-11, which plays a critical role in tumour progression and metastasis, is a direct

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target of miR-124a. Several studies have shown that IL-11 is upregulated in BM samples from liver cancer patients and can promote liver cancer metastasis, thereby affecting the prognosis of patients (22-25). Furthermore, we discovered that the overexpression of miR-124a in HepG2 cells suppresses cell proliferation, inhibits cell migration, and promotes cell apoptosis through the repression of IL-11. These findings could provide new views for the clinical treatment and prevention of the development of liver cancer.

Materials and methods

Cell culture. The human liver cancer cell line, HepG2, was obtained from American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in RPMI-1640 medium (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) containing 10% foetal bovine serum (FBS; Gibco; ThermoFisher Scientific, Inc., Waltham, MA, USA) at 37˚C in a humidified 5% CO_2 atmosphere. miR-124a mimics and negative controls (NCs) were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). The sequences are as follows: miR-124a mimics, 5'-UAAGGCACGCGGUGAAUGCC-3' and 5'-CAUUCACCGCGUGCCUUAUU-3'; RNA NC, 5'-UUCUCCGAACGUGUCACGUTT-3' and 5'-ACGUGACACGUUCGGAGAATT-3'. Small interfering RNAs against IL-11 (sc-39636) was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). At 24 h prior to experiments, cells were transfected with miRNA-124a mimics, RNA NC or IL-11 siRNA according to the manufacturer's recommendations. After 24 h of incubation, cells were trypsinized and used for experimental assays.

MTT assay. HepG2 cells and cells transfected with miR-124a mimics, NCs or IL-11 siRNA were plated in 96-well plates at $5x10^3$ cells/well. A volume of 20 μl sterile methyl thiazolyl tetrazolium (MTT) was added to the cells in each group every 24 h in triplicate. After another 4 h incubation, the supernatant was removed, and 150 μl dimethyl sulfoxide (DMSO) was added into each well with shaking for 15 min. Absorbance was read at 570 nm on an automatic microplate reader to determine the optical density. The cell proliferation rate was calculated by the optical density of the experimental group divided by that of the blank group. Experiments were performed in triplicate.

Transwell chamber assay. We used transwell inserts (Corning, Beijing, China) to assess cell migration. Cells were subjected to transfection, and a cell suspension was prepared. RPMI-1640 medium with 1% BSA was added to the upper chambers, and the lower chambers were filled with 500 μl RPMI-1640 medium with 5% FBS. Approximately 100 μl of a tumour cell suspension was added to the upper chambers and cultured for 24 h. Then, cells on the lower chamber were stained with methyl violet for 25 min and counted under a light microscope. Ten visual fields were selected randomly, and the assay was performed in triplicate.

Western blotting. Cells transfected with miR-124a mimics or NCs were lysed at an appropriate time, and total protein was extracted with the M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, Inc.). After blocking with 5% non-fat milk in TBS with 0.05% Tween-20 (TBST) for 1 h at room temperature, nitrocellulose membranes were incubated overnight at 4˚C with primary antibody dissolved in 5% bovine serum albumin in TBST. The primary antibodies used in this study were as follows: anti-human-MMP2 (1:1,000, 10373-2-AP; Wuhan Sanying Biotechnology, Wuhan, China), anti-human-MMP9 (1:1,000, 10375-2-AP; Wuhan Sanying Biotechnologya), anti-human-tissue inhibitor of matrix metalloproteinase-2 (TIMP-2; 1:1,000, 17353-1-AP; Wuhan Sanying Biotechnology), anti-human-Caspase-3 (1:500, 19677-1-AP; Wuhan Sanying Biotechnology), anti-human-B-cell lymphoma 2 (Bcl-2; 1:1,000, 12789-1-AP; Wuhan Sanying Biotechnology), anti-human-signal transducer and activator of transcription 3 (STAT3; 1:1,000, 10253-2-AP; Wuhan Sanying Biotechnology), anti-human-p-STAT3 (1:500, AF3293; Affinity, Cincinnati, OH, USA), and anti-human GAPDH (1:5,000, 10494-1-AP; Wuhan Sanying Biotechnology) as an internal control. Membranes were incubated for 1 h at room temperature with secondary anti-rabbit Ig-HRP linked (1:3,000, GAR0072; MultiSciences, Hangzhou, China). Immunoreactive proteins were visualized by enhanced chemiluminescence. Image J software (National Institutes of Health, Bethesda, MD, USA) was used to compare the density of band on a western blot. We used the Gel Analysis method to calculate the density of the protein bands relative to the GAPDH standard band. Then we could make statistical analysis.

Enzyme-linked immunosorbent assay (ELISA). To measure the secretion of IL-11, the supernatants from different groups were collected after transfection and assayed by ELISA kits according to the manufacturer's recommendations (Human IL-11 QuantiCyto ELISA kit EHC128.96; Neobio, Shenzhen, China). The absorbance values at 450 nm were used for analysis. Experiments were performed in triplicate.

Dual-luciferase reporter gene assay. Using the bioinformatics tools at TargetScan (http://www.targetcan.org/), potential targets of miR-124a were predicted. The binding sequences for miR-124a in the 3'untranslated regions (3'UTR) of IL-11 were mutated at positions 150 to 156 from GUGCCUU to CGCAGCA. And then we constructed luciferase reporter vectors for the wild-type and mutant-type of IL-11 3'UTR to verify IL-11 is the direct target of miR-124a. Either miR-124a mimics or NC miRNA were co-transfected with the constructed wild-type or mutant-type luciferase reporter vector into 293T cells (ATCC) using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.). Firefly and renilla luciferase activities were assessed with the Dual-Luciferase Reporter Assay (Promega Corporation, Madison, WI, USA) 48 h after cell transfection. The RLU value for renilla luciferase was divided by the RLU value of firefly luciferase to obtain the final result. Experiments were performed in triplicate.

Flow-cytometric analysis of apoptosis. The apoptotic rates of HepG2 cells were detected using an Annexin-V/FITC Kit (BD Biosciences, San Jose, CA, USA). In brief, HepG2 cells were transfected with miR-124a mimics, NCs or IL-11 siRNA and harvested 48 h after transfection by trypsinization. The cell pellet was resuspended in 500 μl binding buffer, 5 μl Annexin V-FITC, and 5 μl PI after washing in ice-cold PBS. Then, it was incubated for 15 min in

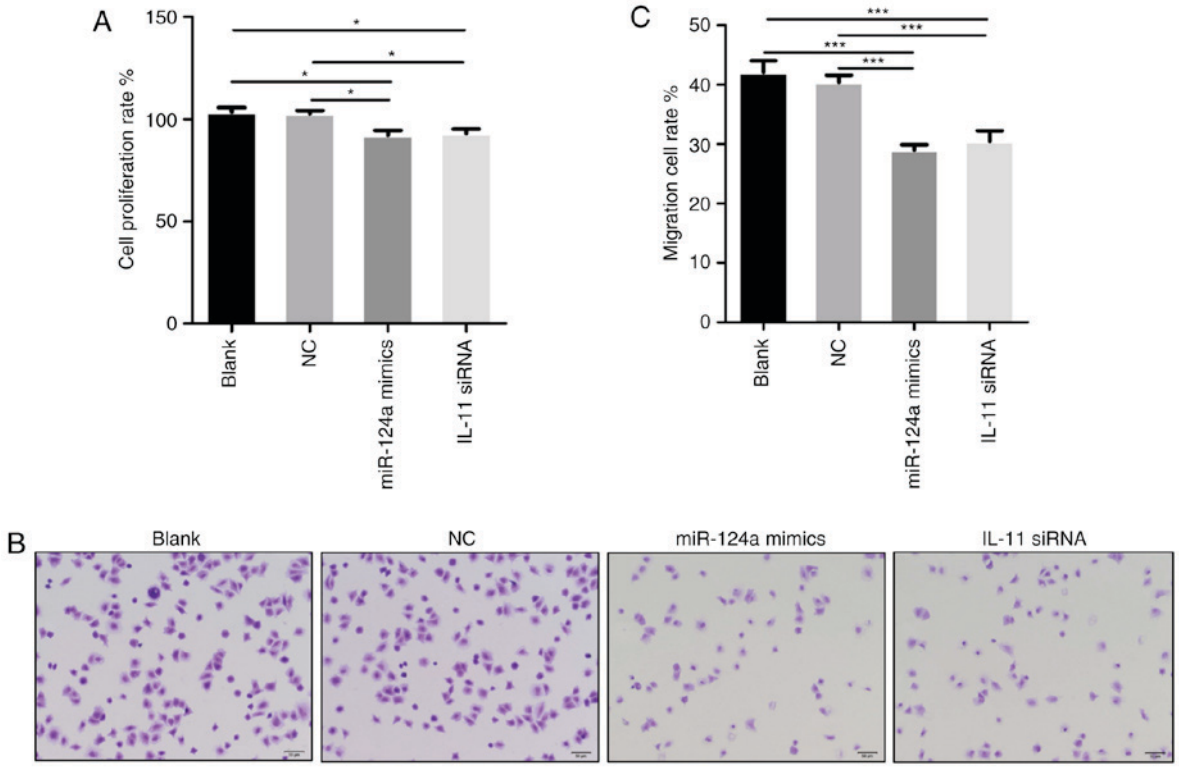

Figure 1. miR-124a inhibits the proliferation and migration of HepG2 cells. (A) The cell proliferation rate of miR-124a mimics and IL-11 siRNA group was much lower than that of the blank and NC group. *P<0.05 vs. blank and NC group. (B) Cells that migrated to the lower chamber were stained with methyl violet and counted under a light microscope (magnification, x100). (C) The upregulation of miR-124a and downregulation of IL-11 can inhibit the migration of HepG2 cells. ***P<0.001 vs. blank and NC group. NC, negative control.

darkness. The ratios of apoptotic cells were determined using a FACSCalibur flow cytometer (BD Biosciences), and data were analysed with FlowJo software. Experiments were performed in triplicate.

Statistical analysis. We used SPSS 19.0 software (SPSS, Inc, Chicago, IL, USA) for statistical analysis. All data are presented as the means ± standard deviation. Comparisons between different groups were analyzed by one-way ANOVA with a Bonferroni post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-124a inhibits the proliferation and migration of HepG2 cells. The proliferation and migration of malignant tumour cells are important prerequisites for tumour development and metastasis. In this study, we used MTT assays and Transwell chamber assays to explore the biological functions of miR-124a by assessing cell proliferation and migration rates. Significantly constrained proliferation of HepG2 cells was observed when cells were transfected with miR-124a mimics or IL-11 siRNA compared with the blank or NC group (Fig. 1A). Cells that migrated to the lower chamber were counted under a light microscope to get the migration rates (Fig. 1B). There were significantly fewer migrated cells in miR-124a mimics and IL-11 siRNA group than in the blank and NC group (Fig. 1C). So, these data indicate that IL-11 is involved in miR-124a-inhibited proliferation and migration.

miR-124a promotes HepG2 cell apoptosis. The regulation of cell apoptosis is an important factor in tumour progression. To determine whether miR-124a and IL-11 could affect the ratios of apoptotic cells, HepG2 cells were divided into the following four groups: Blank, cells transfected with NCs, cells transfected with miR-124a mimics and cells transfected with IL-11 siRNA. Forty-eight h after transfection, the apoptosis of HepG2 cells was assessed (Fig. 2A). Our results reveal that the cell apoptosis rate of miR-124a mimics and IL-11 siRNA was much higher than that of the blank and NC group (Fig. 2B). These results indicate that the upregulation of miR-124a or downregulation of IL-11 can promote HepG2 cell apoptosis, suggesting that IL-11 is involved in miR-124a-regulated cell apoptosis.

IL-11 is a direct target of miR-124a. miRNAs can bind to the 3'UTR of the target gene transcripts to inhibit gene expression. Using the bioinformatics tools at TargetScan (http://www.targetcan.org/), potential targets of miR-124a were predicted. Several results were subsequently analysed by the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (www.genome.jp/kegg/), and IL-11 was evaluated as an effective target of miR-124a. IL-11 contains highly conserved binding sites in the 3'UTR (Fig. 3A). To further confirm that IL-11 is a direct target of miR-124a, DNA fragments containing the wild and mutant IL-11 3'UTR were magnified by PCR and cloned into the luciferase vector psiCHECK-2 to construct the luciferase reporter gene vectors. In order to have higher transfection efficiency to eliminate individual differences in low transfection efficiency, we used 293T cells to conduct dual-luciferase reporter

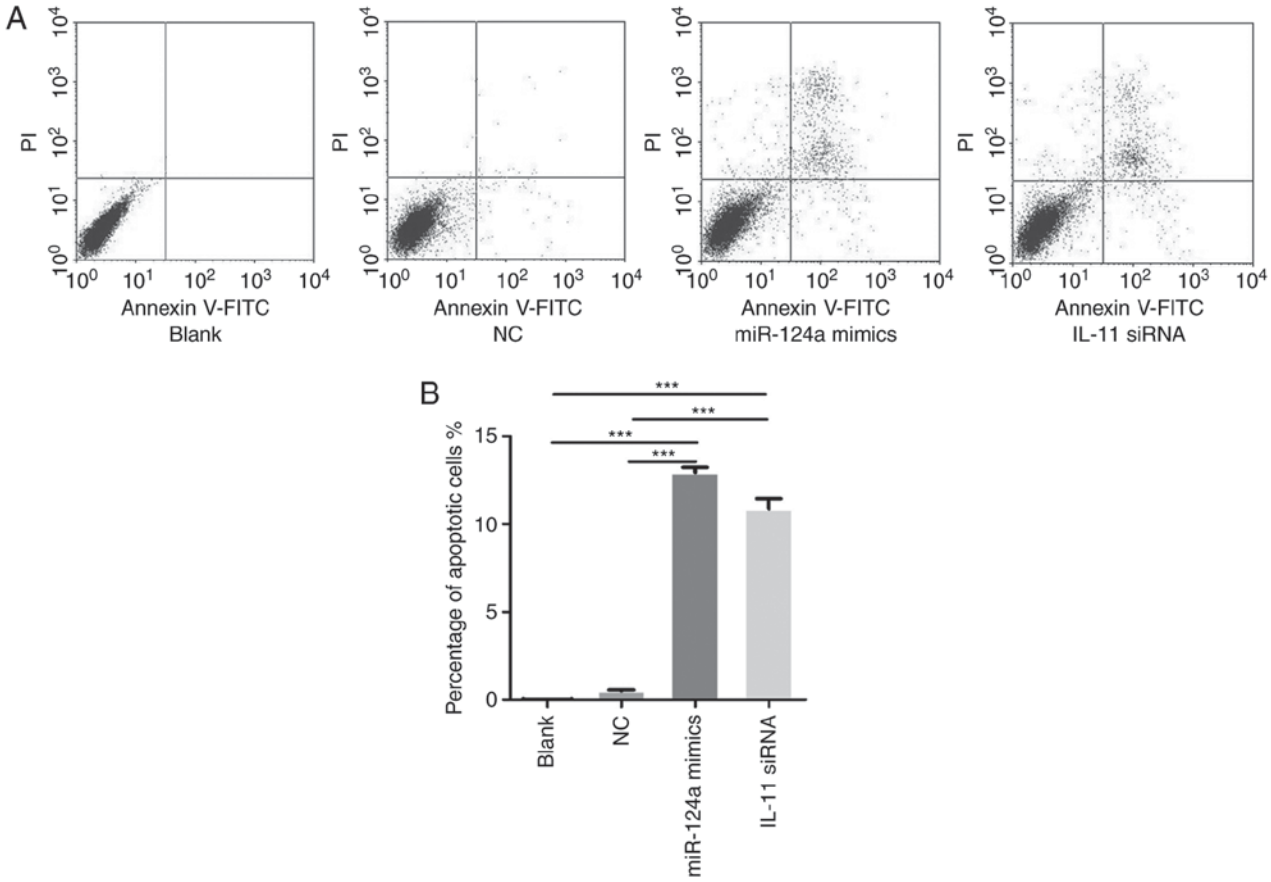

Figure 2. miR-124a promotes HepG2 cell apoptosis. (A) At 48 h after transfection, cells from the various treatment groups were collected, and the HepG2 cell apoptosis rate was altered. (B) The cell apoptosis rate of miR-124a mimics and IL-11 siRNA was significantly higher. ***P<0.001 vs. blank and NC group. NC, negative control.

gene assay. When we transfected either miR-124a mimics or control with wild-type or mutant-type into 293T cells for 48 h, the luciferase activity of IL-11 wild-type with miR-124a mimics was decreased by approximately 40% relative to the miR-124a NC group. However, miR-124a did not significantly affect the luciferase activity of IL-11 with mutant-type 3'UTR (Fig. 3B). ELISA revealed that the secretion of IL-11 was decreased after transfection with miR-124a mimics compared with the blank and NC group (Fig. 3C). These results therefore suggest that miR-124a can bind directly to IL-11 and that IL-11 is a direct target of miR-124a.

Effects of miR-124a on proteins associated with cell proliferation, migration, and apoptosis. We have shown that miR-124a inhibits the proliferation and migration of HepG2 cells and promotes their apoptosis. However, the underlying molecular mechanism remains indefinite. In our study, the expression levels of MMP2, MMP9, TIMP-2, Caspase-3, Bcl-2, STAT3, and p-STAT3 at the protein level were discovered by Western blotting (Fig. 4A). With the upregulation of miR-124a, the expression of MMP2 and MMP9, that are associated with cancer cell metastasis, decreased significantly. In contrast, the expression of TIMP-2, which is an inhibitor of MMP2, increased. The expression of Caspase-3, an important indicator of apoptosis, was significantly upregulated, whereas the expression of Bcl-2, a recognized cell survival factor, was decreased. The apoptotic rate of HepG2 cells increased as a consequence. These results suggest that miR-124a mimics can inhibit metastasis and promote the apoptosis of HepG2 cells. The protein levels of STAT3 and phosphorylated STAT3 (p-STAT3) both significantly decreased, indicating that STAT3 pathway activity was decreased (Fig. 4B).

Discussion

Liver cancer is the third most common cause of cancer-related death due to its high mortality. With continuous improvements in medicine, surgical techniques, and antineoplastic drugs, the five-year survival rate for liver cancer continues to increase. However, because of the high frequency of recurrence and high migratory capacity, the liver cancer patients have a poor prognosis with only a postoperative 5-year survival rate of 30-40%, and the patient's quality of life is often unsatisfactory due to tumour metastasis. Although a variety of tumour suppressor genes and oncogenes have been identified, our understanding of the potential molecular mechanisms in liver cancer metastasis and progression is insufficient. It is necessary to identify and characterize molecular markers that are effective in predicting liver cancer metastasis to establish the prognosis for patients and to determine the most effective therapeutic methods.

Cumulative evidence suggests that the abnormal regulation of microRNAs can contribute to tumourigenesis (26). Changes in miRNA expression are involved in tumour cell progression and metastasis (17,27). Thus, miRNAs are increasingly

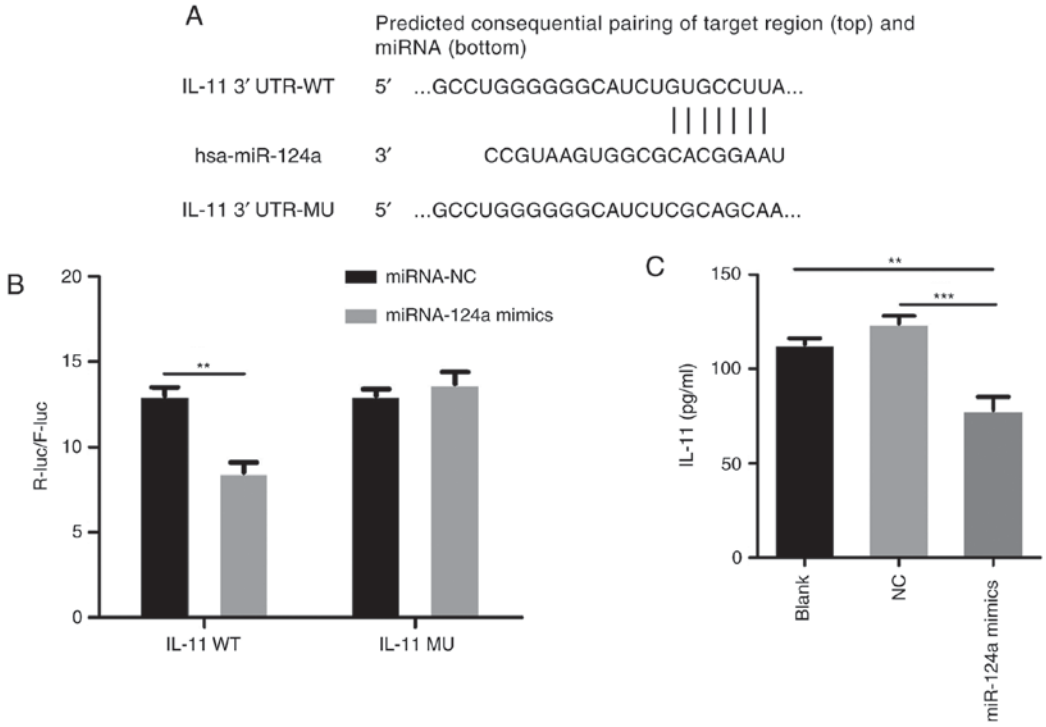

Figure 3. IL-11 is a direct target of miR-124a. (A) The binding of miR-124a to IL-11 predicted by TargetScan. (B) Dual-luciferase reporter gene assays showed that miR-124a markedly inhibited the luciferase activity of IL-11 wild-type relative to the NC group. **P<0.01 vs. NC group. (C) ELISA revealed the different levels of IL-11 secretion among the miR-124a mimic, blank, and NC groups. **P<0.01 vs. blank group, ***P<0.001 vs. NC group. IL, interleukin; NC, negative control.

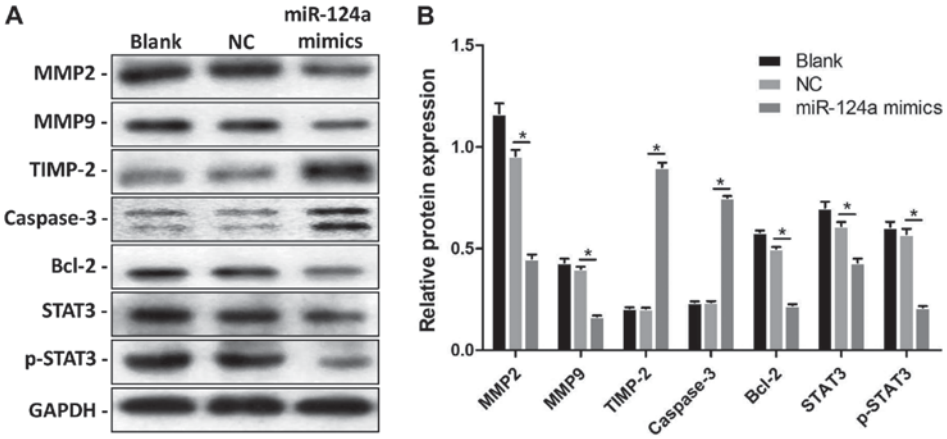

Figure 4. Effects of miR-124a on proteins associated with cell proliferation, migration, and apoptosis. (A) MMP2, MMP9, TIMP-2, Caspase-3, Bcl-2, STAT3, and p-STAT3 protein levels were detected by Western blotting, and GAPDH as an internal control. (B) Relative protein expressions of MMP2, MMP9, TIMP-2, Caspase-3, Bcl-2, STAT3, and p-STAT3 in the miR-124a mimic group and respective negative groups. *P<0.05 vs. NC group. MMP, matrix metalloproteinase; TIMP-2, tissue inhibitor of matrix metalloproteinase-2; Bcl-2, B-cell lymphoma 2; STAT3, signal transducer and activator of transcription 3; NC, negative control.

considered as potential diagnostic and therapeutic targets (28). In the present study, we reviewed the literature and found that miR-124a is downregulated in liver cancer tissues, including hepatocellular carcinoma, cholangiocarcinoma and hepatoblastoma (21,26,27). Meanwhile, miR-124a has been reported to inhibit tumour metastasis in several cancers, including lung cancer (29) and nasopharyngeal carcinoma (30). However, the effects of miR-124a in liver cancer metastasis and its biological mechanism are not fully understood. First, to illuminate the role of miR-124a in hepatoma cells, HepG2 cells were transfected with miR-124a mimics. As is well known, the migration, proliferation, and apoptosis evasion of tumour cells are essential prerequisites for tumour development and metastasis. In our study, miR-124a was upregulated through the successful implementation of miR-124a mimics in HepG2 cells. miR-124a mimics promoted cell apoptosis and inhibited cancer cell proliferation and migration. Knockdown of IL-11 by siRNA suppressed the proliferation, migration and promoted apoptosis of HepG2 cells. These results suggest that IL-11 is involved with miR-124a-regulated HepG2 cell metastasis. Furthermore, we would like to explore the underlying biological mechanism in the miR-124a-mediated inhibition of tumour metastasis.

A key part of tumour migration and metastasis is the degradation of extracellular matrix (ECM) components. MMPs can degrade ECM, and the MMP activity is regulated by TIMPs. Many studies have confirmed that MMP expression is higher in human primary liver cancer and liver metastases compared to normal liver tissue, and some studies have shown relationships between MMP expressions and tumour progression, migration, and metastasis, indicating an important role of MMPs in tumour malignancy (31-33). In this study, the upregulation of miR-124a increased TIMP-2 expression and reduced MMP2 and MMP9 expression in HepG2 cells. Thus, it can be speculated that miR-124a could inhibit the migration of tumour cells by targeting the TIMP/MMP pathway.

To study the correlations of the apoptotic features with relevant signalling molecules, the classical apoptotic genes Caspase-3 and Bcl-2 were analysed by Western blotting. Cell apoptosis is related to the activation, expression, and regulation of many apoptotic genes, among which the family of Bcl-2 and Caspases are widely recognized. Bcl-2 is a member of regulator proteins and acts as an anti-apoptotic protein by inhibiting cell death (34,35). Caspase-3 make great contributions to the implementation stage of cell apoptosis and is widely involved in many pathological processes, including tumourigenesis, infection, trauma, and autoimmune disease (36,37). HepG2 cell line with wildtype p53 was used in our study. The p53 protein could induce apoptosis by activating the expression of pro-apoptotic genes as well as inhibiting the expression of anti-apoptotic genes. It is reported that miR-124 could directly target inhibitor of apoptosis-stimulating protein of p53 (iASPP) and subsequently induce an upregulation of p53 in some tumours, leading to the apoptosis promotion. In the present study, miR-124a mimics promoted Caspase-3 expression and inhibited Bcl-2 expression in HepG2 cells. These results imply that miR-124a can promote apoptosis by promoting Caspase-3 expression and blocking Bcl-2 expression.

IL-11 is located on chromosome 9 and belongs to the IL-6 cytokine family. It has been confirmed that IL-11 plays a significant role in the progression of pancreatic (38), gastric (39), and renal cancers (40) and promotes the BM of liver cancer (41). STAT signalling plays a significant role in the transfer of extracellular signals into the nucleus, resulting in the regulation of transcription, and is essential in the uncontrolled growth, angiogenesis, and metastasis of cancer cells (42,43). STAT3 is a direct target of miR-124 and is overexpressed in most hepatocellular carcinomas with poorer prognosis, however it is not overexpressed in normal liver tissues or in surrounding non-tumour tissues (44,45). Without IL-11/STAT3 signalling, tumour onset is delayed in mice (46). In the present study, the dual-luciferase reporter assay verified that IL-11 is a direct target of miR-124a, and we observed decreased secretion of IL-11 in miR-124a mimic-transfected HepG2 cells. Knockdown of IL-11 by siRNA suppressed the proliferation of HepG2 cells. Moreover, the activity of the STAT3 pathway was decreased, indicating that miR-124a can repress liver cancer proliferation and metastasis by disrupting IL-11/STAT3 signalling.

The organ colonization of diffuse tumour cells can be promoted by IL-11 mRNA, and activating STAT3 signalling (47). Meanwhile, the upregulation of intratumoural IL-11 has been associated with BM after hepatectomy and is an independent prognostic factor for the progress of BM in liver cancer patients (41,48). These studies suggest that IL-11 is closely related to liver cancer BM. According to the inhibitory effects of miR-124a on IL-11, miR-124a may not only inhibit hepatoma cell proliferation, migration, and invasion but also act as a target to repress the BM of liver cancer. However, cumulative evidence has suggested that miR-124 could inhibit metastatic potential of liver cancer, by directly targeting integrin αV, ROCK2 and EZH2 (49,50). So, further researches are required to investigate the potential functional targets of miR-124a, and identifying the actual mechanisms by which miR-124a affects the progression and metastasis of liver cancer requires further clarification.

In summary, we determined that miR-124a can inhibit cell proliferation and migration by directly targeting IL-11, indicating that IL-11 is an important functional mediator of miR-124a in hepatoma cells. These findings may help in investigating potential mechanisms and provide new insights for the clinical treatment to prevent the development and metastasis of liver cancer.

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