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Research article

MiR-23b-3p suppresses epithelial-mesenchymal transition, migration, and invasion of hepatocellular carcinoma cells by targeting c-MET

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HIGHLIGHTS

- c-MET is a target of miR-23b-3p and overexpressed in HCC tissues.
- MiR-23b-3p expression was decreased in HCC tissues compared to non-tumor tissues.
- Inhibition of miR-23b-3p induced migration and invasion via EMT in HCC cells.
- MiR-23b-3p/c-MET axis may serve as a therapeutic target for HCC.

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ABSTRACT

Background: Aberrant expression of c-MET is known to be associated with tumor recurrence and metastasis by promoting cell proliferation, epithelial-mesenchymal transition (EMT), and migration in hepatocellular carcinoma (HCC). Recently, miR-23b-3p has been identified as a tumor suppressor, but detailed role of miR-23b-3p in HCC is still unclear. Our study aimed to investigate how miR-23b-3p is associated with the malignant potential of HCC cells.

Methods: HCC tissues and their adjacent non-tumor tissues were acquired from 30 patients with HCC. Expression of EMT- or stemness-related genes were examined in the two HCC cell lines. Migration of HCC cells was analyzed using transwell and wound healing assays.

Results: c-MET was overexpressed in HCC tissues compared to the adjacent non-tumor tissues. c-MET knockdown inhibited EMT and reduced migration and invasion of HCC cells. Furthermore, c-MET was a target of miR-23b-3p, and miR-23b-3p expression was decreased in HCC tissues compared to non-tumor tissues. Treatment of miR-23b-3p inhibitor in HCC cells promoted EMT, cell migration, and invasion. In contrast, miR-23b-3p overexpression suppressed EMT, cell migration, and invasion, concomitantly reducing c-MET expression. Transfection of miR-23b-3p inhibitor with concomitant c-MET knockdown mitigated the effects of miR-23b-3p inhibitor on EMT in HCC cells. In addition, transforming growth factor beta1 (TGF-β1) stimulation after miR-23b-3p overexpression induced neither the mesenchymal phenotype nor migratory property of HCC cells.

Conclusion: In this study, we confirmed that miR-23b-3p downregulation significantly increased EMT, migration, and invasion of HCC cells. In addition, c-MET was confirmed to be a target of miR-23b-3p in HCC cells and regulated the functional effects of miR-23b-3p. These results suggest that miR-23b-3p can be used as a prognostic biomarker and candidate target for HCC treatment.

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1. Introduction

Hepatocellular carcinoma (HCC) is the most common form of primary liver cancer, and its incidence is increasing worldwide [1, 2]. Despite the availability of various modalities for treatment, including surgery, liver transplantation, and target therapies, the survival rate of HCC patients is still low due to high rate of recurrence and metastasis [3, 4]. Recurrence and metastasis are closely related to epithelial-mesenchymal transition (EMT) in HCC [5, 6]. EMT is characterized by the loss of epithelial markers, such as E-cadherin, followed by upregulation of mesenchymal markers, such as N-cadherin, and strongly promotes cell migration and invasion in most cancers as the initial step of metastasis [7, 8, 9]. It is well established that transforming growth factor-β1 (TGF-β1) is a major inducer of EMT and plays a key role in tumor progression and metastasis in HCC [10]. There is a need for the development of more accurate prognostic predictions and novel targets for the treatment of HCC metastasis.

MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression by binding 3′UTR of target mRNAs and function as oncogenes or tumor suppressors in the development and progression of various human cancers [11]. They are involved in various biological processes in carcinogenesis, such as tumor initiation, development, and metastasis [12, 13]. Among various miRNAs, miR-23 is known to be tightly associated with liver diseases [14]. miR-23 comprises miR-23a and miR-23b, and these two miRNAs differ by only one nucleotide; miR-23a is located on chromosome 19, while miR-23b is located on chromosome 9 [15]. Among them, miR-23b has been shown to be downregulated in several cancers including HCC [16, 17], suggesting that miR-23b-3p may function as a tumor suppressor.

Previously, we reported that dual expression of CD44 and TGF-β1 enhances EMT and migration than single expression of CD44 or TGF-β1 in HCC cells [18]. SNU-368 ($CD44^+$/$TGF\text{-}\beta1^+$) cells were more tightly associated with the metastatic potential of HCCs from its synergy with CD44 and TGF-β1 than SNU-354 ($CD44^+$/$TGF\text{-}\beta1^-$) cells. Based on these results, two cell lines (SNU-354 and SNU-368) were selected to screen potent miRNAs regulating EMT and migration. We selected miR-23b-3p as a biomarker for the regulation of EMT in two HCC cell lines. Furthermore, we identified mesenchymal-epithelial transition factor (c-MET) as a candidate target for miR-23b-3p using target prediction tools. In general, miRTarBase and miRDB are frequently used to predict the interactions between miRNAs and target genes; however, a probability of false predictions exists. To overcome such false predictions, clinical samples may be utilized for the validation of miRNA-mRNA relationships [19]. Considering this, in this study, we used 30 HCC tumor samples to validate the relationship between c-MET and miR-23b-3p to reduce the possibility of false predictions while using the TargetScan program.

The purpose of this study is to show that miR-23b-3p or c-MET may play a role in suppressing EMT, migration, and invasion of HCC, as well as to analyze the interactions between miR-23b-3p and c-MET in the context of EMT and migration. Therefore, in this study, we investigated the role of miR-23b-3p in suppressing HCC migration and invasion by attenuating EMT through the control of c-MET.

2. Materials and methods

2.1. miRNA sequencing with cell lines

To screen differential miRNA expression between control cells and TGF-β1-treated cells, Illumina small-RNA next-generation sequencing (NGS) was performed (Macrogen, Seoul, Korea). Briefly, total RNA was extracted from cells using TRIzol reagents (Invitrogen, Carlsbad, CA, USA), and the quality of RNA samples was confirmed using TruSeq small RNA library prep kit (Illumina San Diego, CA, USA). Then, the miRNA sequencing was loaded out at Illumina HiSeq 2500.

2.2. HCC sample collection

HCC tissues and their adjacent non-tumor tissues were acquired from 30 patients with HCC at Seoul St. Mary's Hospital, Catholic University of Korea (Seoul, South Korea) between June 2018 and April 2021. This study was approved by the Institutional Review Board (IRB) of Seoul St. Mary's Hospital, Catholic University of Korea, and written informed consent was obtained from all the patients (IRB approval number KC17TNSI0484). Clinical characteristics of the enrolled patients are described in Supplementary Table S1.

2.3. Cell culture and treatments

The human HCC cell lines SNU-354, SNU-368 and Huh7 were purchased from Korean Cell Line Bank (KCLB; Seoul, Korea), and HepG2, Hep3B and SK-HEP-1 cells were purchased from ATCC. SNU-354, SNU-368 and Huh7 cells were cultured in RPMI-1640 medium (Welgene, Gyongsan, Korea) containing 10% fetal bovine serum (FBS; Gibco, USA) and 1% antibiotic (Gibco). SK-HEP-1 cells was cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) containing 10% FBS and 1% antibiotic. HepG2 and Hep3B cells were cultured in Minimum Essential Medium (MEM; Gibco) containing 10% FBS and 1% antibiotic. All cells were maintained at 37 °C in 5% CO2. SNU-354 cells were treated with 5 ng/mL TGF-β1 (R&D Systems, Minneapolis, MN, USA) for 48 h, and SNU-368 cells were treated with 1 μM TGF-β inhibitor (SB431542; Selleckchem, Houston, TX, USA) for 24 h.

2.4. Cell transfection

The miR-23b-3p mimic, inhibitor, and corresponding controls were purchased from GenePharma (Shanghai, China). c-MET siRNA and negative control (NC) were purchased from Dharmacon (Lafayette, CO, USA). SNU-354 and SNU-368 cells were transiently transfected with Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) according to the instructions provided by the manufacturer. After transfection for 48 h, the cells were harvested, and the transfection efficiency was analyzed by qRT-PCR.

2.5. RNA extraction and quantitative real-time PCR

RNA was extracted from the cell lines using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) was used to synthesize cDNA to evaluate the expression level of miRNAs. We set up the miRNA cDNA synthesis program on the thermal cycler with the following setting conditions: 16 °C for 30 min, 42 °C for 30 min, 85 °C for 5 min, and hold at 4 °C. PCR was run on the Roche Light Cycler L480 (Roche Applied Science, Indianapolis, IN, USA) for 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s.

QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) was used to synthesize cDNA to assess the mRNA levels. We set up the mRNA cDNA synthesis program on the thermal cycler with the following setting conditions: 42 °C for 30 min, 95 °C for 3 min, and hold at 4 °C. PCR was run on the Roche LightCycler 480 (Roche Applied Science, Indianapolis, IN, USA) for 95 °C for 10 min, followed by 55 cycles of 95 °C for 10 s, 60 °C for 30 s, 72 °C for 1 s. Relative expression levels were normalized to those of U6 snRNA (001973) or GAPDH (Hs02786624) as endogenous controls. The following TaqMan probes were used: hsa-miR-23b-3p (000400), c-MET (Hs01565584), Snail (Hs00195591), Slug (Hs00161904), twist (Hs01675818), Nanog (Hs02387400) and KLF4 (Hs00358836). The relative expression levels were determined using the $2^{-\Delta\Delta Ct}$ method. Total RNA from human normal liver tissue was purchased from Thermo Fisher (AM7960).

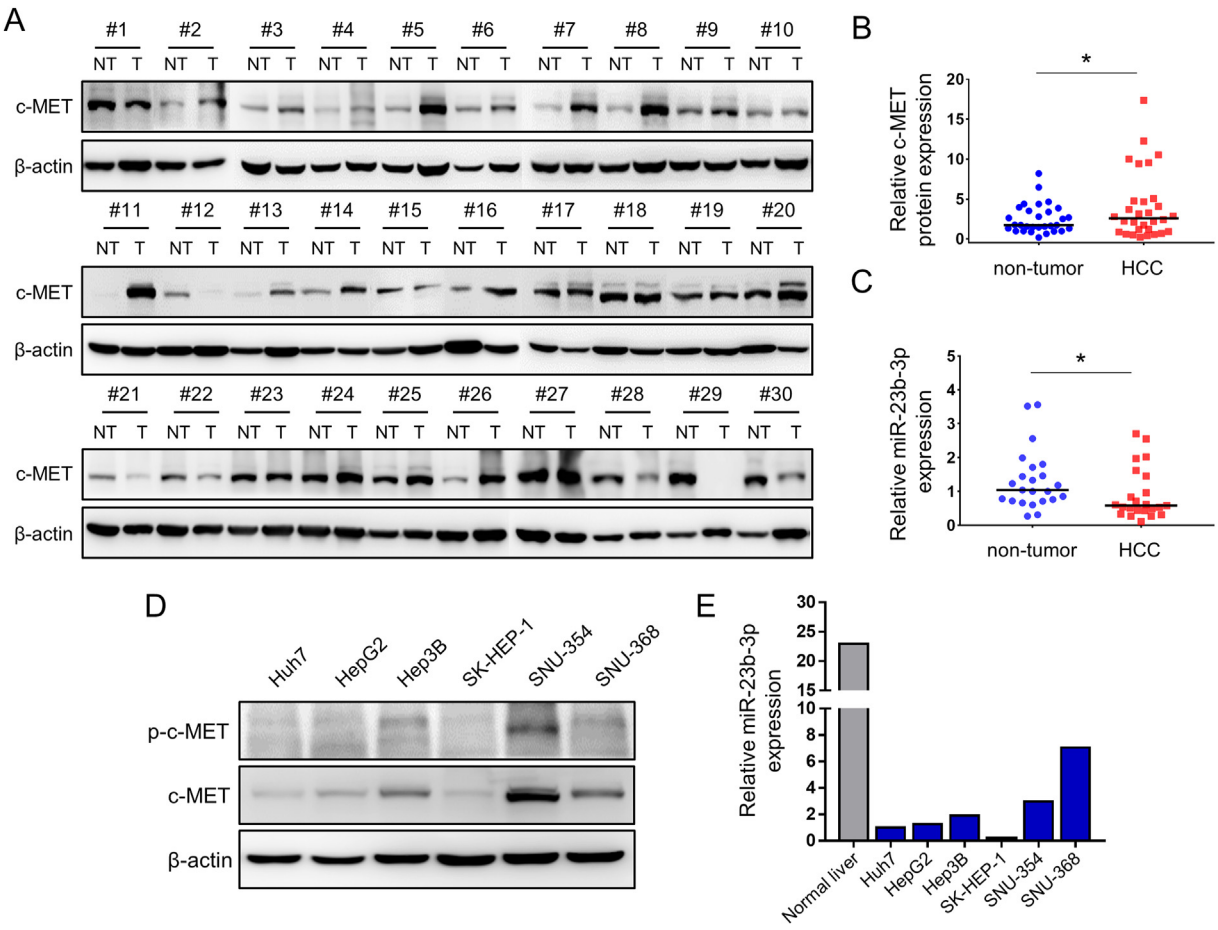

Figure 1. Expression of c-MET and miR-23b-3p in human HCC tissues and cell lines. (A) Western blot analysis of c-MET expression in 30 paired HCC tissues and their adjacent non-tumor tissues. β-actin was used as an internal control. The original blots are presented in Supplementary Figure 3A. (B) Quantification of c-MET protein expression in HCC and their adjacent non-tumor tissues. (C) miR-23b-3p expression levels were evaluated in 23 paired HCC tissues and their adjacent non-tumor tissues by qRT-PCR. (D) c-MET protein expressions were detected using Western blot in HCC cell lines. The original blots are presented in Supplementary Figure 3B. (E) qRT-PCR was used to analyze miR-23b-3p expression in human normal liver and HCC cell lines. The data represent means ± SD *p < 0.05.

2.6. Western blot

Proteins from the cells were lysed using the PRO-PREP protein extraction solution (iNtRON Biotechnology, Seoul, Korea). Equal concentrations of proteins were separated by SDS-PAGE and transferred to PVDF membranes. Then, 5% skim milk or BSA (bovine serum albumin) was used to block the membranes for 60 min at room temperature and the membranes were incubated overnight at 4 °C with the primary antibodies. The next day, membranes were washed thrice and incubated with specific secondary antibodies for 1 h at room temperature. Protein bands were detected using EZ-Western Lumi La (Dogen, Seoul, Korea) and analyzed using the LAS-4000 (Fuji-Film, Tokyo, Japan) imaging system. The following primary antibodies were used: c-MET (1:1000; Cell Signaling, 8198), Claudin-1 (1:1000; Invitrogen, 71-7800), E-cadherin (1:1000; Cell Signaling, 3195), CD44 (1:500; Cell Signaling, 3570), N-cadherin (1:1000; BD Biosciences, 610921), β-catenin (1:1000; Enzo Life Sciences, ALX-804-260), Fibronectin (1:1000; Abcam, ab2413), GAPDH (1:1000; cell signaling, 2118S) and β-actin (1:10000; Sigma-Aldrich, A5441).

2.7. Transwell migration and invasion assay

Transwell chambers uncoated (for migration assay) or coated (for invasion assay) with Matrigel (Corning Incorporated, Corning, NY, USA) were used according to the manufacturer's protocol. To measure the migration and invasive ability of cells, transfected cells were seeded into each upper chamber in serum-free media, and the lower chamber was filled with a complete medium supplemented with 5% or 10% FBS-containing media. The inserts of the invasion chamber were used after rehydration in a serum-free medium for 2 h before the cells were seeded. After 24 or 48 h of incubation, non-migrating or non-invading cells that remained on the top of the transwell insert were removed using a cotton swab. The migratory and invasive cells were then stained with Diff-Quick solution (Sysmex, Japan) and counted.

2.8. Wound healing assay

Transfected cells were seeded onto 6-well plates. The next day, the cells were scratched using a sterile 200 μL pipette tip. The culture medium was replaced with a fresh 0.5% FBS-containing medium. The wound closure was photographed at 0 and 48 h under a microscope.

2.9. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 7 (GraphPad software, San Diego, CA, USA) and SPSS 20.0 software (IBM, Armonk, NY, USA). All data were presented as mean ± standard deviation (SD) or median. Comparisons between two groups were evaluated using two-tailed Student's t-test or Mann-Whitney U-test. The experiments were performed at least in triplicate. Statistical significance was set at $*p < 0.05$, $**p < 0.005$, $***p < 0.0005$.

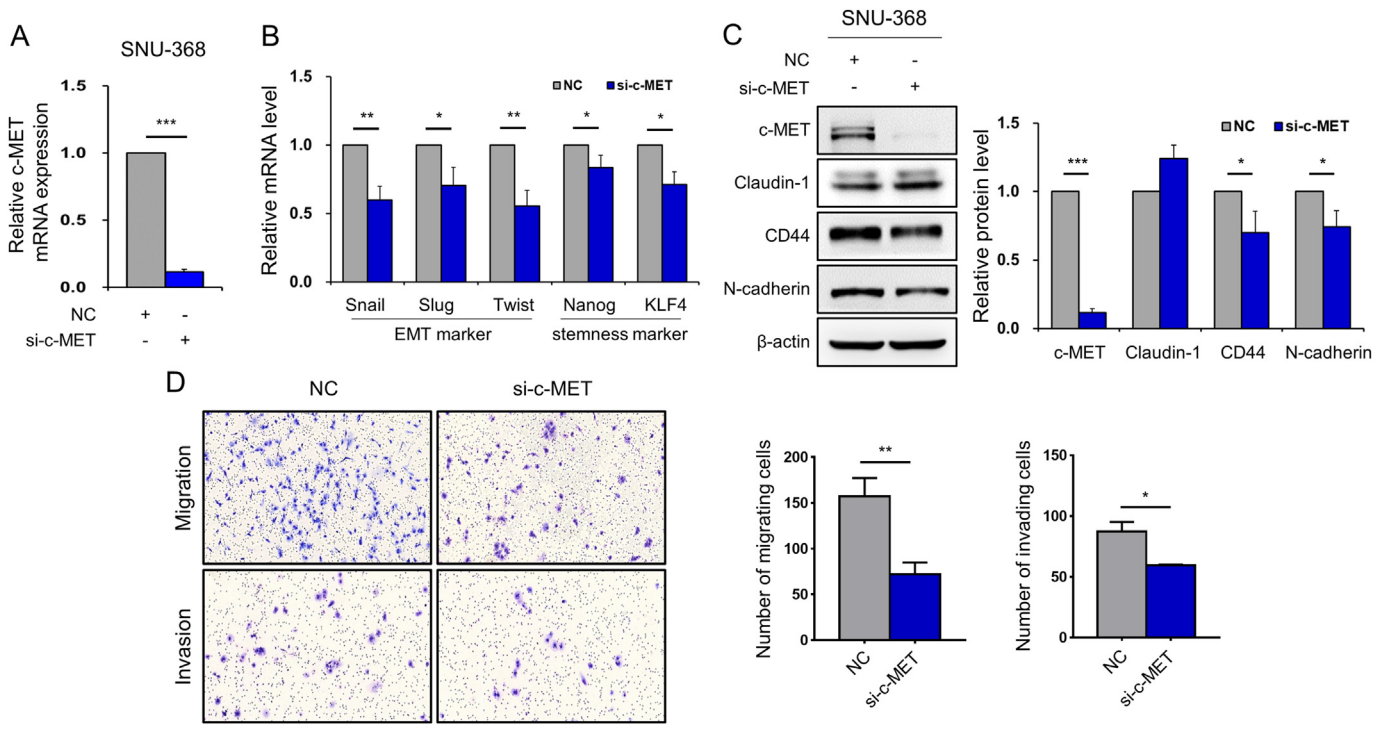

Figure 2. Knockdown of c-MET suppresses EMT, migration, and invasion of HCC cells. (A) Expression levels of c-MET after transfection with c-MET siRNA and negative control were analyzed by qRT-PCR. GAPDH was used as an internal control. (B and C) Expression levels of EMT and stemness-related genes after transfection with c-MET siRNA and negative control were analyzed by qRT-PCR (B) and Western blot (C). GAPDH and β-actin were used as an internal control. NC, negative control. The original blots are presented in Supplementary Figure 3C. (D) Transwell migration and invasion assays of SNU-368 cells transfected with c-MET siRNA and negative control (NC) using a transwell plate with 8 μm pores without or with matrigel (magnification, ×100). Representative images (left) and quantitative data (right) of the transwell experiment. The data represent means ± SD *p < 0.05, **p < 0.005, and ***p < 0.0005 compared with control.

3. Results

3.1. High c-MET expression correlates with low miR-23b-3p expression in human HCC tissues and cell lines

To identify miRNAs related to EMT, small-RNA next-generation sequencing (NGS) was performed using control cells and TGF-β1-treated cells. As a result, upregulation of two miRNAs and downregulation of seven miRNAs were noted in TGF-β1-treated cells. Of these, miR-23b-3p was selected because the other miRNAs were not reliably detected in HCC cells by qRT-PCR. Considering that miR-23b-3p acts as a tumor suppressor miRNA in HCC, all further analyses in this study focused on miR-23b-3p (Supplementary Figure 1A). Moreover, according to the prediction by using bioinformatics tools, c-MET was considered a candidate target for miR-23b-3p. Thus, we confirmed the expression pattern of c-MET and miR-23b-3p in HCC tissue and cell lines. c-MET was analyzed in 30 paired HCC and adjacent non-tumor specimens by western blotting (Figure 1A). The protein levels of c-MET were significantly higher in HCC tissues than in the adjacent non-tumor tissues by Mann-Whitney U-test (Figure 1B). On the other hand, the expression of miR-23b-3p in HCC tissues was lower than that in adjacent non-tumor tissues (Figure 1C). Moreover, c-MET was upregulated in HCC cell lines (Figure 1D), while miR-23b-3p expression was decreased in HCC cell lines compared to human normal liver (Figure 1E).

3.2. Knockdown of c-MET suppressed EMT, migration, and invasion of HCC cells

Our previous study confirmed that SNU-368 ($CD44^+$/TGF-$\beta1^+$) cells readily undergo EMT and have migratory property compared to SNU-354 ($CD44^+$/TGF-$\beta1^-$) cells via the synergy between CD44 and TGF-β1 [18]. c-MET siRNA was transfected into SNU-368 cells, and knockdown efficiency was determined by qRT-PCR (Figure 2A) and Western blot analysis (Figure 2C). c-MET knockdown decreased the mRNA expression levels of EMT transcription factors Snail, Slug, and Twist (Figure 2B). In addition, the protein expression levels of the mesenchymal marker N-cadherin were markedly reduced, and the protein expression levels of the epithelial marker Claudin-1 were increased (Figure 2C). Moreover, the mRNA expression levels of stemness-associated genes, Nanog and KLF4, were significantly downregulated in c-MET knockdown cells (Figure 2B). CD44 protein also showed similar results in these cells (Figure 2C). Finally, transwell assay showed that c-MET knockdown suppressed the migration and invasion of SNU-368 cells (Figure 2D). Collectively, these data confirmed that c-MET knockdown blocks EMT, migration, and invasion of HCC cells.

3.3. c-MET is a target of miR-23b-3p in HCC cells

To validation the results of NGS, we induced EMT by TGF-β1 treatment or inhibited EMT by TGF-β1 inhibitors. TGF-β1-stimulated SNU-354 cells exhibited EMT induction through the loss of E-cadherin expression and gain of CD44, N-cadherin, β-catenin, Snail, and Slug expression (Figure 3A and B). In comparison, TGF-β1 inhibition in SNU-368 cells blocked EMT through increased E-cadherin and decreased CD44, N-cadherin, β-catenin, Snail, and Slug expression (Figure 3D and E). The expression of miR-23b-3p was downregulated by TGF-β1 stimulation (Figure 3C) and upregulated by TGF-β1 inhibitor (Figure 3F). These results suggest that miR-23b-3p may act as a tumor suppressor in HCC and that downregulation of miR-23b-3p is involved in TGF-β1-induced EMT.

To explore the molecular mechanisms of HCC progression by miR-23b-3p, we used two different bioinformatics tools (miRTarBase and miRDB) to predict the putative target genes of miR-23b-3p and identified c-MET as a potential target. Binding sites of miR-23b-3p and c-MET were

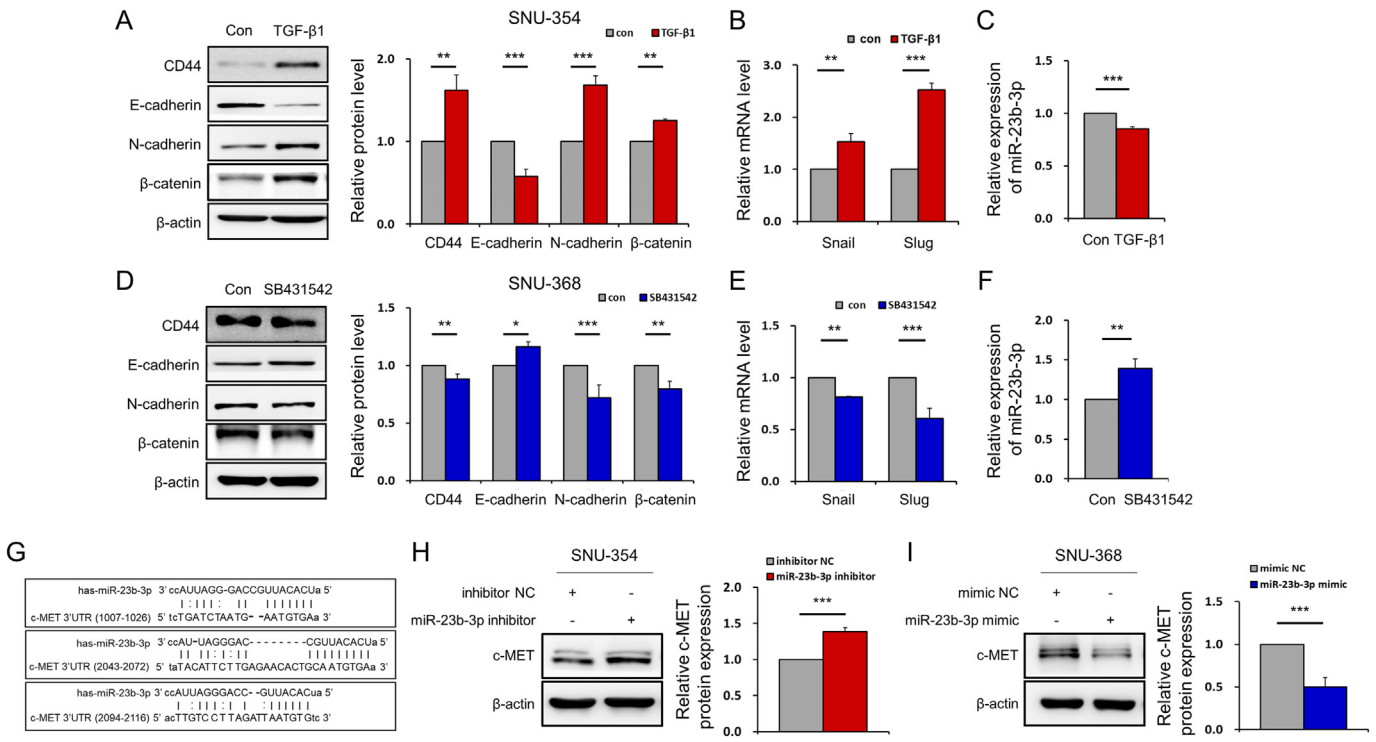

Figure 3. c-MET is a direct target of miR-23b-3p. (A and B) Expression levels of EMT-related markers in SNU-354 cells treated with TGF-β1 were determined by Western blot analysis and qRT-PCR. β-actin and GAPDH were used as an internal control. The original blots are presented in Supplementary Figure 3D. (C) Expression level of miR-23b-3p after TGF-β1 stimulation was analyzed by qRT-PCR and normalized to U6 snRNA. (D & E) Expression levels of EMT-related markers in SNU-368 cells treated with a TGF-β1 inhibitor SB431542 were determined by Western blot analysis and qRT-PCR. The original blots are presented in Supplementary Figure 3E. (F) miR-23b-3p expression after SB431542 treatment was analyzed by qRT-PCR. (G) The binding sites between miR-23b-3p and c-MET were predicted by bioinformatics analysis. (H and I) miR-23b-3p inhibitor was transiently transfected into SNU-354 cells (H), and miR-23b-3p mimic was transfected into SNU-368 cells (I). Protein expression level of c-MET was determined using Western blot. The original blots are presented in Supplementary Figure 3F and G. The data represent means ± SD *p < 0.05, **p < 0.005, and ***p < 0.0005 compared with control.

predicted with miRTarBase (Figure 3G). In addition, miR-23b has been reported to target c-MET [20, 21, 22]. We investigated whether miR-23b-3p regulates c-MET expression in two HCC cell lines by western blotting after miR-23b-3p inhibitor and mimic transfection. Inhibition of miR-23b-3p significantly increased c-MET protein expression in SNU-354 ($CD44^+$/TGF-$\beta1^-$) cells, whereas overexpression of miR-23b-3p significantly decreased c-MET protein expression in SNU-368 ($CD44^+$/TGF-$\beta1^+$) cells (Figure 3H and I). Collectively, these results confirmed that c-MET is a potential target of miR-23b-3p and that miR-23b-3p regulated c-MET expression.

HGF is known to activate c-MET, and we investigated the effects of miR-23b-3p on c-MET activation by HGF. In the presence of 25 or 50 ng/mL HGF, HGF activated c-MET and decreased the expression of miR-23b-3p in a dose-dependent manner (Supplementary Figure 2A and B). Moreover, transfection of miR-23b-3p mimic inhibited c-MET activation in HGF-treated SNU-354 cells (Supplementary Figure 2C).

3.4. Overexpression of miR-23b-3p blocked EMT, migration and invasion of HCC cells

To investigate the functional impact of miR-23b-3p on EMT, SNU-354 cells were transiently transfected with an miR-23b-3p inhibitor, and SNU-368 cells were transfected with miR-23b-3p mimics. We confirmed the transfection efficiency of miR-23b-3p in the mimic and inhibitor groups by qRT-PCR. miR-23b-3p levels were decreased in SNU-354 cells transfected with miR-23b-3p inhibitors and increased in SNU-368 cells transfected with miR-23b-3p mimics compared to those in the negative control (Figure 4A and B). After 48 h of transfection, downregulation of miR-23b-3p in SNU-354 cells decreased the expression of the epithelial marker Claudin-1 and increased the expression of the stem-cell marker CD44 and the mesenchymal markers N-cadherin, β-catenin, and Fibronectin (Figure 4C). In comparison, upregulation of miR-23b-3p in SNU-368 cells significantly decreased the expression of the CD44 and N-cadherin, β-catenin, and Fibronectin (Figure 4D). These results revealed that inhibition of miR-23b-3p promoted EMT and overexpression of miR-23b-3p inhibited EMT in HCC cell lines. However, expression of the epithelial marker E-cadherin, which is predicted to be directly regulated by miR-23b-3p, showed a trend different from that of Claudin-1 (Figure 4C and D).

We evaluated the effect of miR-23b-3p on the invasive capacity of SNU-354 and SNU-368 cells using migration, invasion, and wound healing assays. As expected, inhibition of miR-23b-3p strongly enhanced transwell migration/invasion and cell motility in SNU-354 cells compared to the negative control (Figure 4E and F). In contrast, the overexpression of miR-23b-3p strongly reduced transwell migration/invasion and cell motility in SNU-368 cells compared to the negative control (Figure 4G and H). Taken together, these results indicate that miR-23b-3p regulates cell migration and invasion capabilities of HCC cells.

3.5. c-MET is involved in the process of EMT by miR-23b-3p in HCC cells

To confirm the relationship between c-MET and miR-23b-3p expression, SNU-354 cells were co-transfected with an miR-23b-3p inhibitor in the presence or absence of c-MET knockdown. Transfection with miR-23b-3p inhibitor induced EMT; however, c-MET knockdown mitigated this EMT-promoting effect (Figure 5A). Moreover, c-MET knockdown dramatically reversed the effects of migration and invasion caused by miR-23b-3p inhibition (Figure 5B). These results demonstrate that c-MET is involved in the process of EMT by miR-23b-3p in HCC cells.

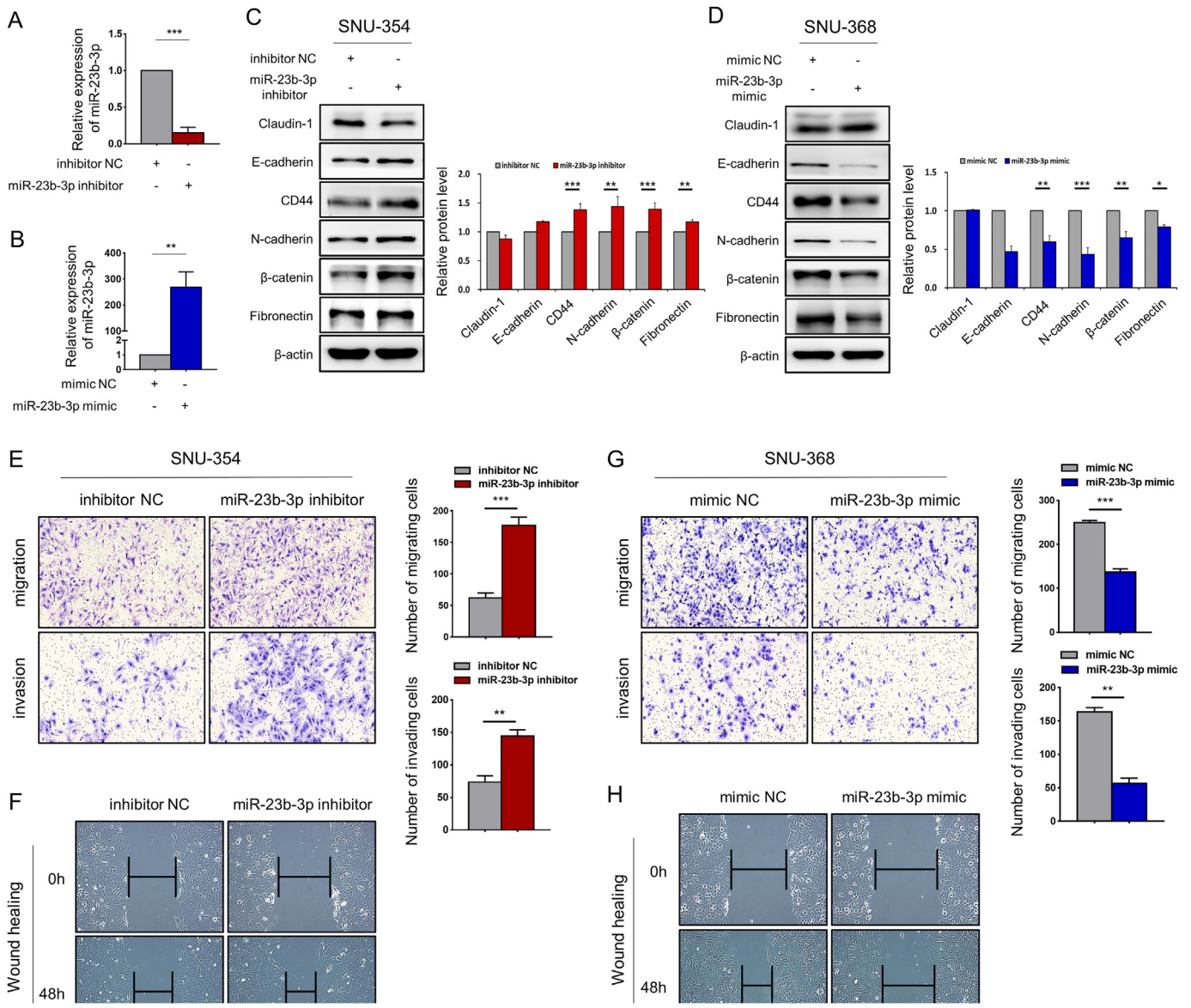

Figure 4. Overexpression of miR-23b-3p suppressed EMT, migration and invasion of HCC cells. (A and B) Transfection efficiency of miR-23b-3p inhibitor in SNU-354 cells and miR-23b-3p mimic in SNU-368 cells were analyzed by qRT-PCR. U6 snRNA was used as an internal control. (C and D) Western blot detected the protein level of Claudin-1, E-cadherin, CD44, N-cadherin, β-catenin, and Fibronectin in two HCC cell lines after transfection with an miR-23b-3p inhibitor, miR-23b-3p mimic, and the respective negative controls (NCs). β-Actin was used as an internal control. The original blots are presented in Supplementary Figure 3F and G. (E and G) Transwell migration and invasion assays of cells transfected with miR-23b-3p inhibitor, mimics and the corresponding negative control (NC) were performed using a transwell plate with 8 μm pores without or with matrigel (magnification, ×100). The representative images and quantitative data of the transwell experiment. (F and H) Wound healing assays were performed after transfection with miR-23b-3p inhibitor and mimic. Wound areas were measured at 0 h and 48 h. The data showed represent ±SD *$p < 0.05$, **$p < 0.005$, and ***$p < 0.0005$ compared with control.

3.6. *miR-23b-3p blocks TGF-β1-induced EMT in HCC cells*

Next, we investigated whether miR-23b-3p could inhibit TGF-β1-induced EMT, migration, and invasion. To confirm that miR-23b-3p suppresses TGF-β1-induced EMT, an miR-23b-3p mimic was transfected into SNU-354 cells treated with TGF-β1 (Figure 6A). As shown in Figures 3 and 6, SNU-354 cells downregulated the expression of epithelial markers and upregulated the expression of mesenchymal marker by TGF-β1 treatment (Figure 6B and C). In addition, the expression of the stemness marker CD44 also increased (Figure 6C). Overexpression of miR-23b-3p in TGF-β1-treated HCC cells led to the reverse EMT with loss of mesenchymal and stemness markers and upregulation of epithelial markers except E-cadherin (Figure 6C). Consistent with these findings, miR-23b-3p overexpression strongly suppressed TGF-β1-induced cell migration and invasion (Figure 6D). Taken together, our results indicate that miR-23b-3p overexpression counteracted the effects of TGF-β1-induced EMT and migration in HCC cells.

4. Discussion

HCC is a common malignant tumor with high recurrence and metastasis rates and is complicated by multiple lesions. miRNAs regulate proliferation, apoptosis, invasion, EMT, and drug resistance during the progression of HCC. The identification of cancer-related miRNAs and their target genes is necessary for the development of diagnostics and therapeutics for HCC. Several studies have shown that EMT can lead to epithelial cell trans-differentiation into mesenchymal cells, leading to cancer cell metastasis [6]. Our current study identified miR-23b-3p as an

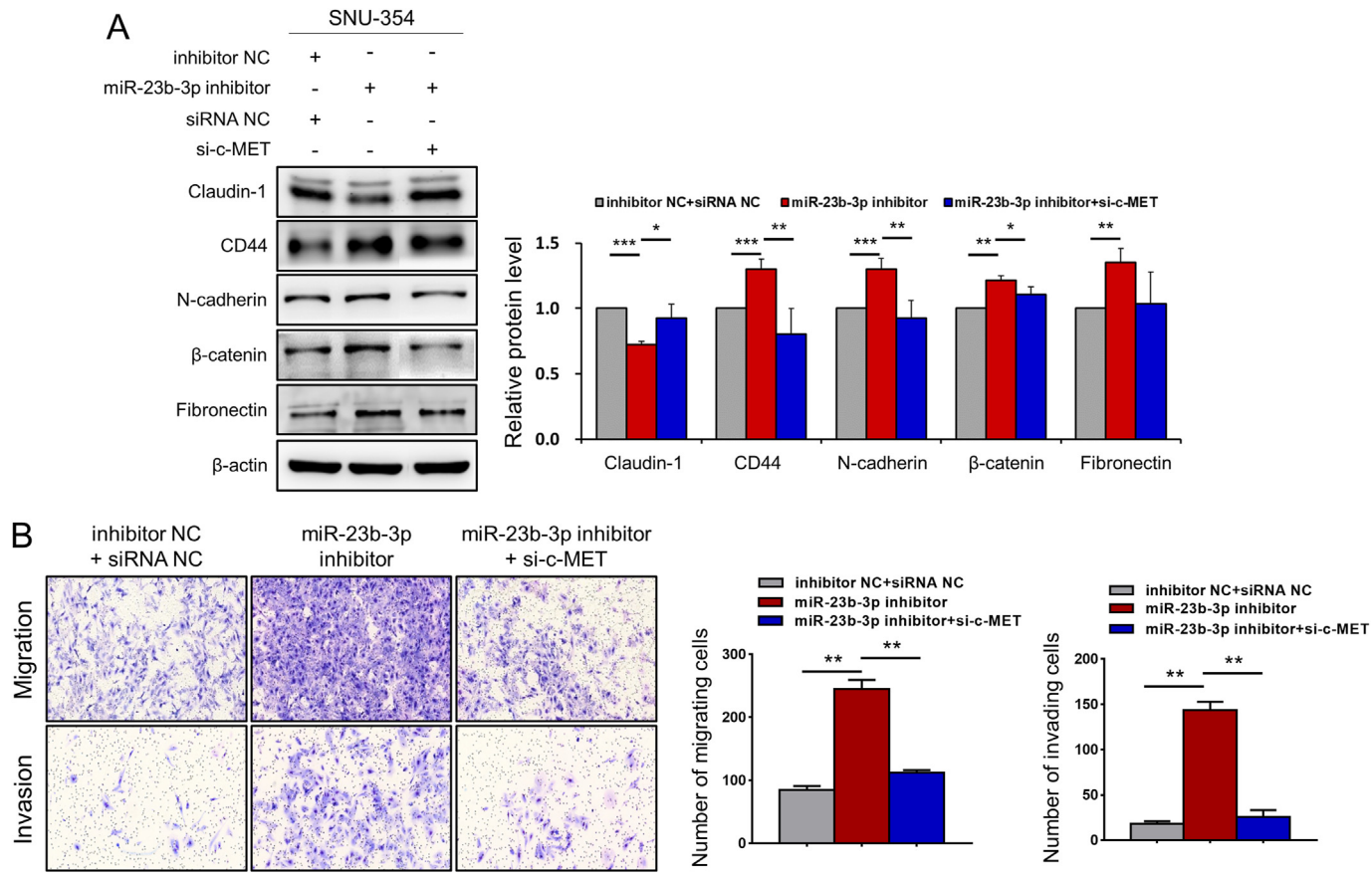

Figure 5. c-MET is involved in the process of EMT by miR-23b-3p in HCC cells. (A) SNU-354 cells were transfected with miR-23b-3p inhibitor and co-transfected with or without c-MET siRNA. Then, the EMT-related protein was examined by Western blot. β-actin was used as an internal control. The original blots are presented in Supplementary Figure 3H. (B) Migration and invasion in the SNU-354 cells transfected with miR-23b-3p inhibitor alone or co-transfected with c-MET siRNA were assessed by transwell assays. The representative images and quantitative data of the transwell experiment. The data represent means ± SD *$p < 0.05$, **$p < 0.005$, and ***$p < 0.0005$ compared with control.

anticancer factor in TGF-β1-induced EMT models. Downregulated miR-23b-3p promotes HCC progression, and its reduction is associated with poor prognosis in patients with HCC [16].

Although some studies have reported that miR-23b may decrease the migration of HCC cells by targeting c-MET, the definite mechanism of action of the interactions between miR-23b-3p and c-MET during EMT has not been fully elucidated [20]. Interestingly, this study was developed based on the findings of previous studies, to screen for potent miRNAs that regulate EMT. SNU-354 ($CD44^+$/TGF-$β1^-$) cells were treated with TGF-β1, while SNU-368 ($CD44^+$/TGF-$β1^+$) cells were treated with SB431542 (TGF-β1 inhibitor). Among miRNAs, miR-23b-3p expression was distinctly downregulated during the process of TGF-β1-induced EMT and was notably upregulated in reverse EMT by SB431542 (Figure 3). In addition, miR-23b-3p overexpression reversed the effects of TGF-β1-induced EMT and migration (Figure 6). Further, we found that miR-23b-3p regulates both c-MET and CD44, which is associated with EMT. It is important to gain an in-depth understanding of the mechanism involved in the regulation of EMT by tumor-suppressive miR-23b-3p that regulate metastasis-promoting genes to obtain a successful therapeutic outcome in HCC. Hereby we elucidated the anti-metastatic role of miR-23b-3p as an important regulator that modulates EMT and migration by targeting c-MET in HCC cells.

miR-23b-3p plays dual roles as a tumor suppressor and tumor inducer by regulating several genes in various human cancers [23]. For example, miR-23b is downregulated in colorectal cancer (CRC) cells and primary CRC tissues compared to non-malignant colorectal tissues, and is associated with poor prognosis in patients with CRC [24]. CB1R-induced tumor progression in gastric cancer may be suppressed by miR-23b-3p [25]. miR-23b-3p modulates the progression of cervical cancer and acts as a tumor suppressor by targeting c-MET [26]. Consistent with these findings, our study showed that miR-23b-3p is downregulated in HCC tissues and miR-23b-3p overexpression blocks EMT and invasive activity in HCC cells. These data revealed that miR-23b-3p might exhibit anti-cancer activity and act as a key regulator of metastasis in HCC cells. Furthermore, the bioinformatics analysis identified several potential downstream targets of miR-23b-3p, including c-MET, GSK3β, CDH1, CD44, and ELK3. Among the targets, the results of our study showed that miR-23b-3p regulated the expression of c-MET, thereby affecting EMT and migration in HCC cells.

c-MET is a receptor tyrosine kinase activated by binding to hepatic growth factor (HGF) [27]. In general, c-MET is essential for embryonic development and regeneration [28, 29, 30]. However, aberrant c-MET activation can promote the development and progression of tumors [31]. Correspondingly, the inhibition of c-MET has a significant impact on the reduction of cell proliferation, EMT, migration, and metastasis. Accumulating evidence has shown that overexpression of c-MET promotes the proliferation, survival, and metastasis of tumor cells and leads to poor prognosis in HCC patients [32, 33, 34]. Therefore, activation of HGF/c-MET affects multiple events from tumorigenesis to metastasis and may be an important therapeutic target in HCC [29]. In this study, c-MET was a direct target of miR-23b-3p, as identified by bioinformatic analysis, and transfected miR-23b-3p negatively regulated the expression of c-MET. Furthermore, we found that c-MET silencing may not only restrain EMT, but also downregulates stemness genes, including CD44, Nanog, and KLF. We showed that c-MET silencing inhibited the migration and invasion of HCC cells (Figure 2). More importantly, EMT induction in

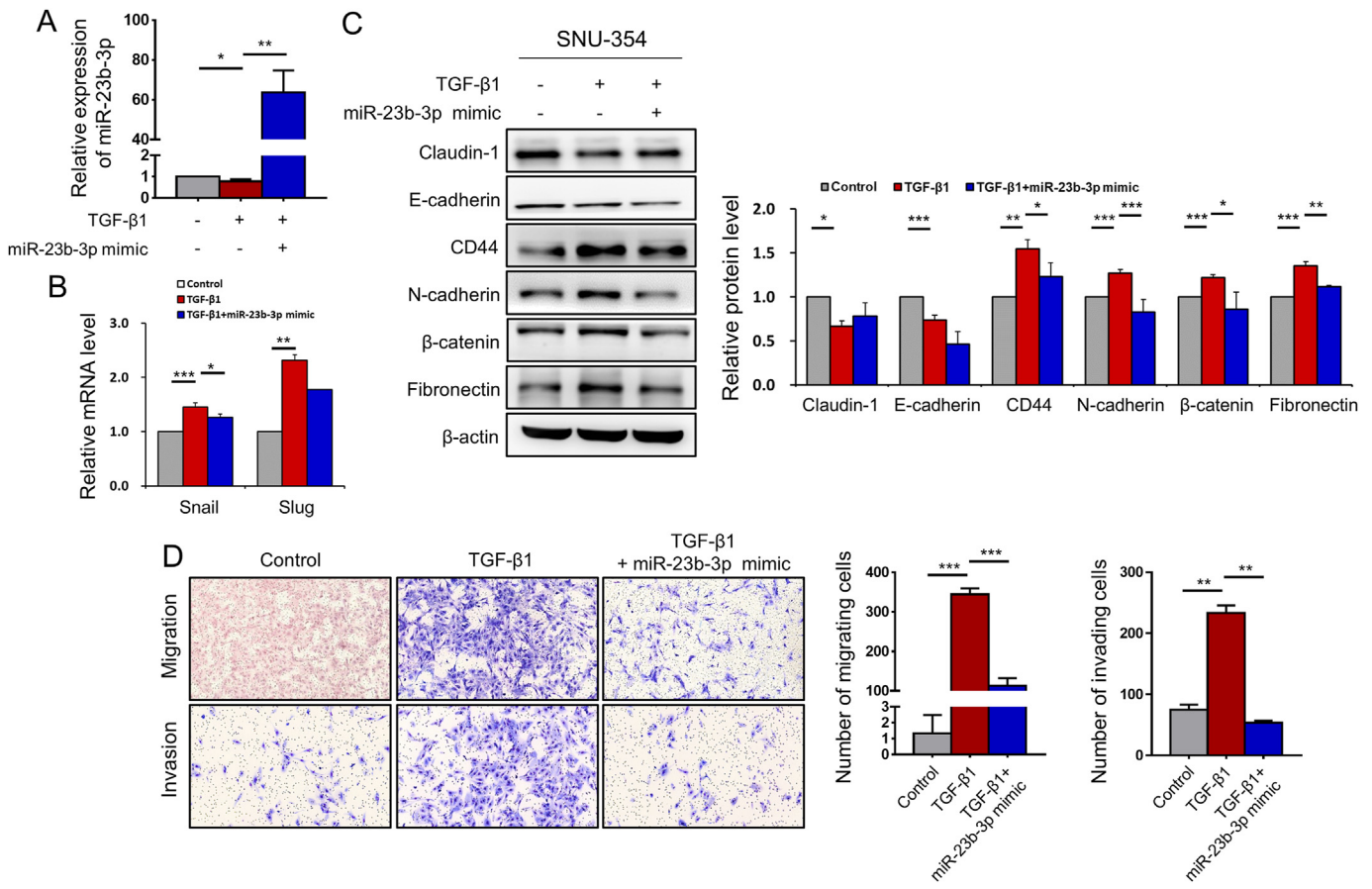

Figure 6. miR-23b-3p blocks TGF-β1-induced EMT in HCC cells. (A) SNU-354 cells were transfected with miR-23b-3p mimics and then treated with or without 5 ng/mL of TGF-β1 for 24 h. qRT-PCR confirmed the expression levels of miR-23b-3p. (B and C) The EMT-related mRNA and protein levels were examined using qRT-PCR (B) and Western blot (C). GAPDH and β-actin were used as an internal control. The original blots are presented in Supplementary Figure 3I. (D) Transwell migration and invasion assays in the SNU-354 cells treated with TGF-β1 alone or transfected with miR-23b-3p mimics and treated with TGF-β1 were assessed by transwell assays. The representative images and quantitative data of the transwell experiment. The data represent means ± SD *p < 0.05, **p < 0.005, and ***p < 0.0005 compared with control.

HCC cells, which was caused by miR-23b-3p inhibition, was also rescued by c-MET silencing (Figure 5). Collectively, we confirmed that miR-23b-3p regulates malignant potential of HCC cells by targeting c-MET.

CD44 has been widely studied as a surface biomarker of cancer stem cells and a critical regulatory factor in EMT [35]. CD44 is involved in several biological processes such as tumor initiation, development, and metastasis. Previous studies have shown that CD44 promotes EMT and migration in HCC cell lines and that the synergy of CD44 and TGF-β1 makes an increase in metastatic potential. Based on these findings, this study demonstrated that blocking the function of miR-23b-3p regulates CD44 expression by inducing EMT through the activation of c-MET. As CD44 is also a direct target of miR-23b-3p, our results indicate a potent anti-metastatic function of miR-23b-3p by targeting two major genes involved in EMT, the c-MET and CD44. The mutual regulation between miR-23b-3p and its target gene in this loop could more clearly tune gene expression in EMT-related cancer metastasis. Therefore, we speculated that miR-23b-3p could potently prevent HCC metastasis by repressing both c-MET and CD44.

Furthermore, HCC is an inflammation-associated tumor, and recurrence after treatment often amplify its immunosuppressive state. Several studies have shown that miRNAs contribute to immune system development, response, and program activation, and can act as oncogenes or tumor suppressors by modulating immunological responses involved in cancer-associated pathways [36, 37, 38]. Therefore, the concept of immuno-miRNAs in HCC has attracted the attention of most researchers and will be an innovative therapeutic approach for HCC through its crucial role in immune response as well as oncogenic and antitumor pathways. Unfortunately, studies on miRNAs based on the activation or suppression of immune responses in HCC are in early stages. Further investigations pertaining to the role of miRNAs, including miR-23b-3p, in the immunotherapeutic response of liver cancer, their ability to regulate the immune response, and the related mechanisms are warranted. In addition, cabozantinib, a small-molecule inhibitor of c-MET and VEGFR2, has proven its efficacy in a phase III trial for patients with advanced HCC treated with sorafenib. The researchers observed a lower risk of death in the treatment group compared to the placebo group when considering the overall survival and progression-free survival of patients with HCC [39]. According to our analyses, the combination therapy of miR-23b-3p and the c-MET inhibitor cabozantinib can increase the treatment efficacy for patients with advanced or recurrent HCC; however, detailed future studies are required.

Our study has the following limitations. First, in vivo validation of our observations should be performed. Second, more human HCC samples with different treatment modalities are needed to validate the suitability of miR-23b-3p as a potential biomarker for HCC treatment. Lastly, detailed mechanisms how c-MET regulates the EMT in HCC cells should be investigated further. Nevertheless, our results demonstrate that miR-

23b-3p is an attractive therapeutic target for suppressing HCC metastasis by potently regulating EMT through c-MET inhibition.

5. Conclusions

In conclusion, we confirmed that miR-23b-3p downregulation significantly increased EMT, migration, and invasion of HCC cells, whereas overexpression of miR-23b-3p exerted the opposite effect. miR-23b-3p overexpression strongly suppressed TGF-β1-induced EMT and invasive activity. In addition, c-MET was confirmed to be a target of miR-23b-3p in HCC cells and regulated the functional effects of miR-23b-3p, including EMT, migration, and invasion. We have demonstrated that miR-23b-3p possesses antimetastatic properties by inhibiting EMT and migration by regulating c-MET in HCC cells. Thus, miR-23b-3p may act as a potentially reliable therapeutic target for EMT-related cancer metastasis. Future research will demonstrate the roles of specific miRNAs in regulating the responses to immunotherapy for HCC.

Declarations

Author contribution statement

Na Ri Park: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Jung Hoon Cha: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Pil Soo Sung: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Jeong Won Jang: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Jong Young Choi; Seung Kew Yoon: Analyzed and interpreted the data.

Si Hyun Bae: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Data availability statement

No data was used for the research described in the article.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

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