

Anti-invasion and anti-migration effects of miR-199a-3p in hepatocellular carcinoma are due in part to targeting CD151

JI HYE KIM^{1*}, MOHAMED BADAWI^{1*}, JONG-KOOK PARK^{1,5}, JINMAI JIANG^{1,6},
XIAOKUI MO², LEWIS R. ROBERTS³ and THOMAS D. SCHMITTGEN^{1,4}

¹College of Pharmacy, ²Department of Biostatistics, Ohio State University, Columbus, OH;

³Divisions of Gastroenterology and Hepatology, Mayo Clinic College of Medicine, Rochester, MN, USA

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Abstract. Several studies have reported reduced miR-199a-3p expression in hepatocellular carcinoma (HCC). In an effort to discover important target genes for miR-199a-3p that may be related to HCC development or progression, we identified the tetraspanin, transmembrane glycoprotein CD151. Luciferase reporter assays and western blotting identified CD151 as a *bona fide* miR-199a-3p target gene. While CD151 protein was increased in the mesenchymal but not the epithelial HCC cell lines, CD151 knockdown with siRNA did not reduce HCC cell proliferation in either group of cells. miR-199a-3p reduced *in vitro* invasion and migration of CD151-positive HCC cells. Examination of the mRNA and protein expression in pairs of primary HCC tumors and adjacent benign tissues showed that not only was CD151 mRNA and protein increased in the tumors but also that an inverse correlation exists between the miR-199a-3p and CD151 RNA expression. We report that CD151 is a target of miR-199a-3p and that increased CD151 protein resulting from reduced miR-199a-3p could contribute to the development of metastatic HCC.

Introduction

Hepatocellular carcinoma (HCC) is the second most common cause of cancer death worldwide, accounting for an estimated 745,000 deaths per year, representing 10% of all deaths from cancer (1). Major risk factors for HCC include hepatitis C

virus (HCV) or hepatitis B virus (HBV) infection, alcoholic cirrhosis and nonalcoholic fatty liver disease. Resection, local ablation or transplantation are effective treatments for early stage HCC. Transarterial chemoembolization shows limited success for intermediate stage HCC without invasion or metastasis (2). However, the overall survival for advanced HCC is poor due to rapid tumor progression and metastasis (3). Therefore, it is necessary to better understand the mechanisms of HCC metastasis and to develop new therapeutic options for advanced or recurrent HCC.

It has been shown that expression of miRNAs is dysregulated in all cancers (4). miRNAs may play an oncogenic or tumor suppressive role depending on the type of cancer. miR-199a-3p is a miRNA that displays decreased expression in HCC (5-8). The genes that encode miR-199a-1 and miR-199a-2 are located within introns of the DNMT2 and DNMT3 genes, respectively. Previous research has shown that miR-199a-3p regulates expression of c-MET (9,10), mTOR (9) and PAK4 (6). We previously reported that CD44 is a target of miR-199a-3p in HCC (11).

CD151 (Tspan24) is a member of the tetraspanin protein family that have been linked to metastasis (12-14). CD151 is associated with proMMP7 and proMMP9 transcription which facilitates matrix degradation and regulates cell migration. Several studies have demonstrated that CD151 is involved in the regulation of pathways downstream of the hepatocyte growth factor (HGF)/c-Met axis (15) and CD151 was remarkably overexpressed in HCC (12). High expression levels of CD151 and integrin subunit $\alpha 6$ increased invasiveness of HCC cells (14) and overexpression of CD151 promoted the expression of MMP9, which is one of the key factors in metastasis through the PI3K/Akt/GSK-3 β /Snail pathway (13). Recent studies showed that CD151 expression could be regulated by miRNAs. miR-506 suppressed CD151 in a breast cancer cell line (16) and miR-124 inhibits invasiveness and metastatic potential of breast cancer cells by targeting CD151 mRNA (17). In addition, miR-22 reduces cell proliferation and invasiveness of gastric cancers by suppressing CD151 (18).

We confirm previous findings that low miR-199a-1 expression is correlated with poor survival in HCC and that miR-199a-3p is significantly downregulated in HCC (5-8). We report that CD151 is a direct target of miR-199a-3p and

Correspondence to: Dr Thomas D. Schmittgen, ⁴Present address: College of Pharmacy, University of Florida, Gainesville, FL 32610, USA

E-mail: tschmittgen@ufl.edu

Present addresses: ⁵Department of Dermatology, Northwestern University, Chicago, IL, USA; ⁶College of Pharmacy, University of Florida, Gainesville, FL, USA

*Contributed equally

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that reintroduction of miR-199a-3p to HCC cells strikingly suppressed cell migration and invasion *in vitro* in part by targeting CD151.

Materials and methods

Cell line and tissue specimens. The human HCC cell lines SNU-423, SNU-449, PLC/PRF/5, HepG2, Hep3B and SK-Hep-1, were purchased from American Type Tissue Collection (Manassas, VA, USA). SNU-423, SNU-449 cells were cultured in RPMI-1640 medium (Invitrogen) with 10% fetal bovine serum (Sigma). PLC/PRF/5, Hep3B, HepG2 and SK-Hep-1 were cultured in MEM medium (Invitrogen) with 10% fetal bovine serum (Sigma). All cell lines were successfully authenticated by the Interdisciplinary Center for Biotechnology Research at the University of Florida (data not shown). Eighteen paired HCC and adjacent non-tumor liver tissues were collected from patients during surgical resections at the Mayo Clinic (Rochester, MN, USA), frozen in liquid nitrogen and stored at -80° until RNA and protein were extracted. Sample collection conformed to the policies and practices of the facility's Institutional Review Board. Patient demographics are presented in Table I. The TNM classification for hepatocellular carcinoma was used for tumor stage and grade.

Transfection of microRNA mimic and siRNA oligonucleotides. HCC cell lines were transfected either with 100 nM of hsa-miR-199a-3p mimic or negative control (Ambion), or with 100 nM of CD151 siRNA or control siRNA (Thermo Scientific) using Lipofectamine 2000 (Invitrogen) and Opti-MEM medium (Invitrogen). Cells were transfected with the miRNA mimic or siRNA oligonucleotides for 72 h prior to extraction of RNA or protein.

RNA extraction, cDNA synthesis and qRT-PCR. Total RNA was extracted from 18 pairs of HCC tumors and adjacent benign liver tissue specimens. Following pulverization in a cold mortar and pestle, total RNA was isolated from the tissues using TRIzol reagent (Life Technologies). cDNA was synthesized as previously described (19). Five hundred nanograms of total RNA was used to synthesize cDNA using random primers. cDNA was analyzed for gene expression using gene specific primers (IDT) and the Express SYBR[®] GreenER qPCR super mix (Invitrogen). For the miRNA expression analysis, cDNA primed with 100 ng of total RNA was assayed using the TaqMan[®] microRNA assays (Applied Biosystems) as described (20). Data were normalized to 18S rRNA and the relative expression of genes was presented using the comparative C_T method. Data were multiplied by 10^6 to simplify presentation. Primer sequences are available upon request.

Dual-luciferase reporter gene assay. The full length CD151 3'UTR was cloned into the psiCHECK-2 Vector (Promega). Three nucleotides in the binding sequences of CD151 3'UTR was mutated by QuikChange XL Site-Directed Mutagenesis kit (Agilent Technologies) and the mutation was confirmed by sequencing. The luciferase reporter gene assay was performed using the Dual-luciferase reporter assay system (Promega)

according to the manufacturer's instructions. SNU-449 cells were plated at a density of 50,000 cells/well (24-well). Following a 24-h incubation, cells were co-transfected with miR-199a-3p mimic or negative control oligonucleotide (50 nM) along with the WT-CD151 3'UTR or mutated CD151 3'UTR constructs using Lipofectamine 2000 (Invitrogen). Reporter gene assays were performed 48 h post-transfection using the Dual luciferase assay system (Promega). *Renilla* luciferase activity was normalized for transfection efficiency using the corresponding Firefly luciferase activity. All experiments were performed at least three times.

Cell proliferation assay. PLC/PRF/5, SK-Hep-1, SNU-423 and SNU-449 cells were seeded at a density of 2,000 cells per well in 96-well culture plates. Following a 24-h incubation period, cells were transfected with 100 nM of CD151 siRNA or control siRNA oligonucleotides. Cell proliferation was determined 96 h later using the WST-1 reagent (Roche) per the manufacturer's recommendations. All experiments were performed at least in triplicate.

Cell migration assay. A wound healing assay was performed to evaluate cell migration *in vitro*. SNU-449 cells were transfected with either 100 nM of miR-199a-3p mimic or negative control oligonucleotides, or 100 nM of CD151 siRNA or control siRNA. Twenty-four hours after transfection, 70 μ l of the transfected cells (3×10^5 cells/ml) was placed into each well of an ibidi culture-insert (ibidi, LLC). After an overnight incubation, the culture insert was removed to create a cell-free gap in the monolayer. The gap closure area was photographed and analyzed by TScratch software (21). The percentage of the gap area closed between the beginning and end of the experiment was calculated from at least three independent experiments.

Matrigel invasion assay. *In vitro* cell invasion assays were conducted using the CytoSelect[™] 24-well cell invasion assay kit (8- μ m pore size, Cell Biolabs, Inc.). SNU-449 cells were transfected with either 100 nM of miR-199a-3p mimic or negative control oligonucleotides, or 100 nM of CD151 siRNA or control siRNA. Forty-eight hours after transfection, the transfected cells were placed into the upper chamber at a density of 1.5×10^5 cells per well in 1% FBS containing medium. Ten percent FBS containing medium was placed in the lower chamber as a chemoattractant. Cells were incubated at 37°C for 24 h and the cells that invaded the membrane were fixed and stained. The number of cells invading the membrane were counted in three different fields per experiment.

Protein extraction and immunoblotting. Cell protein lysates in RIPA buffer (Sigma) were separated on NuPAGE 4-12% Bis-Tris gels (Novex) and electrophoretically transferred to polyvinylidene difluoride membranes (Roche). The blotting was performed for CD151 (ab33315, Abcam). β -actin (Abcam) or GAPDH (sc-32233, Santa Cruz Biotechnology) were used as loading controls. Secondary horseradish peroxidase antibody was detected using the ECL Western Blotting Analysis system (Amersham Biosciences).

Statistical analysis. The matched samples were compared using paired t-tests and samples subjected to different treat-

Table I. Patient data.

Patient	Gender	Age	Grade	Stage	Tumor size (cm) (largest mass)	Cirrhosis	Fibrosis stage	Etiology
10	F	62.5	2	IV	22	No	0	No known risk factors
14	M	89.1	2	IV	6.5	No	0	Hereditary hemochromatosis
26	M	43.3	1	I		No	0	No known risk factors
33	F	43.2	2	I		Yes	4	HCV
44	F	48.0	1	I		No	0	No known risk factors
69	F	85.0	1	I		No	0	No known risk factors
92	M	76.2	2	II		Yes	4	Autoimmune hepatitis
94	F	82.7	3	II		No	0	No known risk factors
97	F	50.8	2	I	10.5	No	0	No known risk factors
99	F	83.3	3	I		Yes	4	HCV
103	F	84.7	2	II	8	No	0	Alcohol
106	M	59.9	2	I		No	0	Alcohol
109	M	80.3	2	I	6	No	0	No known risk factors
306	M	72.3	2	II	4.3	Yes	4	No known risk factors
309	M	81.2	3	I	5	No	0	HCV probable remote history HCB
337	M	80.7	3	II	14.4	Yes	4	Alcohol
361	M	71.0	3	I	10.8	No	0	No known risk factors
367	M	71.5	2	II	4	No	2	HCB

ments were compared using a Student's 2-sample t-tests. A $p < 0.05$ was considered significant. The Cancer Genome Atlas (TCGA) microRNA-seq expression data and patients' clinical information ($n=141$) were downloaded through the TCGA data portal. Patients were dichotomized into two groups (high and low) according to the median expression of miR-199-1 or miR-199-2. The probabilities of 5-year survival between groups were compared by using the Kaplan-Meier method and log-rank test. Data analysis was performed using SAS 9.4 (SAS, Inc; Cary, NC, USA). For the analysis of miR-199-1 expression in paired benign and HCC, data from 49 pairs of benign and HCC were used. This represents all of the paired specimen data for HCC on the TCGA data portal.

Results

CD151 is a target of miR-199a-3p in HCC. To better understand the role of miR-199a-3p in HCC, we searched potential miR-199a-3p targets using the TargetScan algorithm. A putative miR-199a-3p binding site within the CD151 3'UTR was highly conserved (Fig. 1A). A luciferase reporter assay was used to confirm the binding of miR-199a-3p to the CD151 3'UTR. Luciferase expression was reduced by >50% by miR-199a-3p mimic (Fig. 1B). The luciferase expression was not reduced substantially when the miR-199a-3p binding site on the CD151 3'UTR was mutated (Fig. 1B). To further investigate if miR-199a-3p functionally regulates CD151, SNU-423 and SNU-449 cells were transfected with miR-199a-3p mimic or negative control oligonucleotides. Western blotting experiments showed that the protein expression of CD151 was reduced by 60% in SNU-423 and by 43% in SNU-449

cells compared to control (Fig. 1C). qRT-PCR showed that miR-199a-3p mimic reduced CD151 mRNA in these cell lines (Fig. 1D). Together these data indicate that CD151 is a direct target of miR-199a-3p and that the miR-199a-3p binding enhances CD151 mRNA degradation.

Attenuation of CD151 does not reduce HCC proliferation. CD151 protein as well as several epithelial-mesenchymal transition (EMT) markers were examined in HCC cell lines. HepG2, Hep3B and PLC/PRF/5 express high levels of CDH1, an epithelial marker whereas SNU-449, SNU-423 and SK-Hep-1 express high levels of the mesenchymal markers VIM, ZEB2 and TWIST (Fig. 2A-D). Interestingly, CD151 protein was overexpressed in the mesenchymal-like cell lines (SNU-449, SNU-423 and SK-Hep-1) compared to cell lines expressing epithelial genes (HepG2, Hep3B and PLC/PRF/5) (Fig. 2E), suggesting CD151 is associated with the mesenchymal phenotype. To determine if a direct relationship exists between mesenchymal markers and CD151, the expression of epithelial (CDH1) and mesenchymal (VIM, CDH2 and ZEB1) was measured in SNU-423 cells following siRNA knockdown of CD151. Knockdown of CD151 by $\leq 80\%$ did not significantly alter the expression of CDH1, VIM, CDH2 or ZEB1 (data not shown).

To determine if CD151 regulates cell proliferation, we transfected four different HCC cell lines with CD151 or control siRNA. Knockdown of CD151 by >80% failed to reduce cell proliferation in either CD151-negative (PLC/PRF/5) or CD151-positive (SK-Hep-1, SNU-423 and SNU-449) cells (Fig. 2F). These results suggest that CD151 is not involved in regulating HCC cellular proliferation.

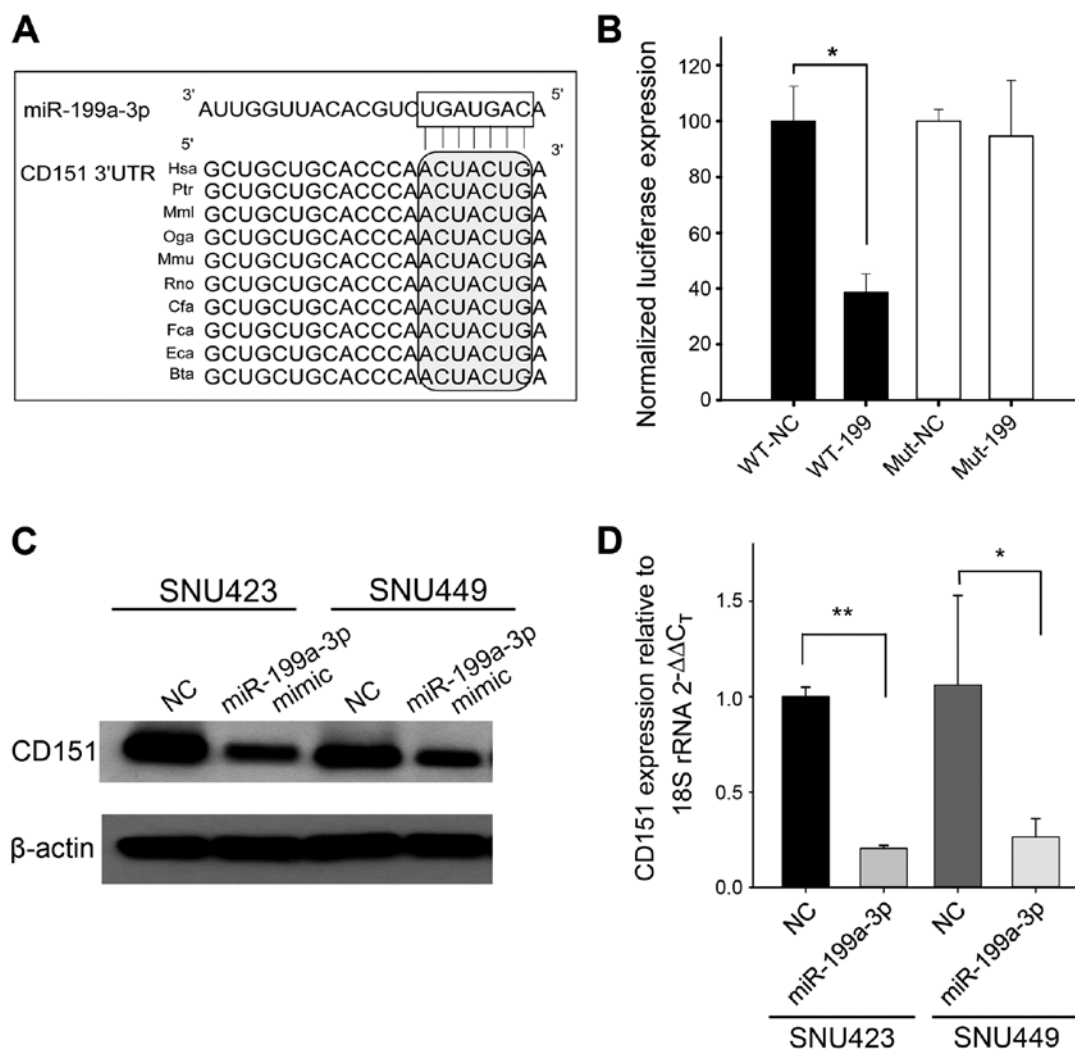


Figure 1. CD151 is a target of miR-199a-3p. (A) Schematic diagram representing the location and conservation of the putative miR-199a-3p binding site within the 3'UTR of CD151 mRNA. (B) Luciferase reporter plasmids containing the wild-type or mutated CD151 3'UTR were transiently transfected into the SNU-449 cell line with 50 nM of miRNA-199a-3p mimic control or miR-199a-3p oligonucleotides. Luciferase expression was measured 48 h after transfection. Normalized *Renilla* luciferase activity in cells transfected with negative control oligonucleotides (NC) was set at 100%. The data represent the mean \pm SD of at least 3 independent transfections. (C) Immunoblot of CD151 protein expression in SNU-423 and SNU-449 cells transfected with control oligonucleotide or miR-199a-3p mimic. (D) Relative CD151 mRNA expression was measured by qRT-PCR in control oligonucleotide or miR-199a-3p mimic transfected cells.

In vitro cell migration and invasion is inhibited by miR-199a-3p through targeting CD151. Next we determined whether miR-199a-3p mimic could inhibit *in vitro* cell migration and invasion under conditions of CD151 suppression. CD151-positive SNU-449 and SNU-423 cells were transfected with miR-199a-3p mimic under the identical conditions shown to suppress CD151 mRNA and protein expression. As a control, wound healing and invasion assays were performed following transfection with CD151 or control siRNA. Compared to control oligonucleotide, wound healing was decreased in SNU-449 cells after transfection of miR-199a-3p mimic (Fig. 3A and B). Wound healing was also significantly reduced after CD151 siRNA transfection compared to control siRNA (Fig. 3C and D). We also repeated the wound healing experiment in a second cell line (SNU-423 cells) with similar results (Fig. 3E). In addition, the number of invading SNU-449 cells was strikingly reduced after transfection with miR-199a-3p mimic (Fig. 4A and B). Cell invasiveness was also significantly suppressed after CD151 siRNA transfection (Fig. 4C and D).

The results were reproduced in SNU-423 cells (Fig. 4E). These data suggest that suppression of CD151 expression by miR-199a-3p mimic can reduce cell migration and invasion *in vitro*.

miR-199a-3p and CD151 expression inversely correlates in HCC specimens. The expression of miR-199a-3p was measured in 18 pairs of human HCC tissues and adjacent benign tissues by qRT-PCR. miR-199a-3p was significantly downregulated in the HCC tissues compared to the adjacent benign liver ($p < 0.0001$, paired t-test, Fig. 5A), confirming previous results (5-8). We also evaluated the expression of miR-199a-1 and miR-199a-2 in 49 pairs of tumor and adjacent benign from the TCGA data set. miR-199a-1 and miR-199a-2 are two isogenic genes encoding miR-199a-3p. The expression of miR-199a-1 (Fig. 5B, $p < 0.0001$) and miR-199a-2 ($p < 0.0001$, not shown) was reduced in the tumor compared to the adjacent benign tissue. Next, we investigated the correlation between miR-199a gene expression and survival by analyzing data from the TCGA database. HCC patients with high miR-199a-1

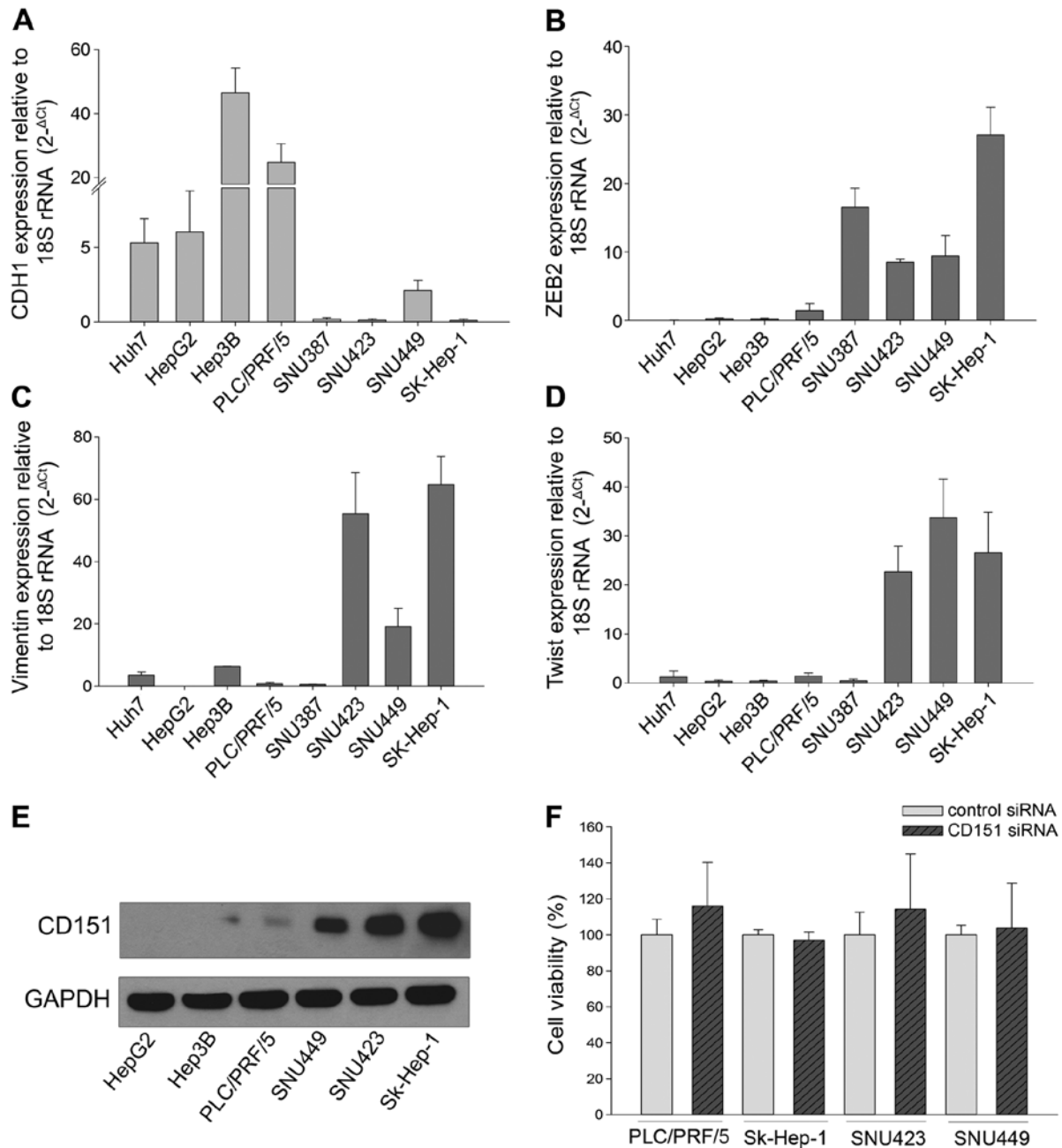


Figure 2. CD151 is overexpressed in mesenchymal HCC cell lines and is not involved in cell proliferation *in vitro*. Several epithelial [(A) CDH1] and mesenchymal [(B) ZEB1, (C) VIM and (D) TWIST] markers were analyzed in HCC cell lines by qRT-PCR. (E) CD151 protein levels were assayed by western blotting in HCC cell lines. (F) HCC cell lines were transfected with 100 nM control siRNA or CD151 siRNA. Proliferation was determined by WST-1 assay 96 h after transfection.

expression had better survival compared to those with low miR-199a-1 levels ($p < 0.05$, Fig. 5C). While there was good separation between survival and miR-199a-2 expression, the correlation was not significant ($p = 0.3$, data not shown).

CD151 mRNA (qRT-PCR) and protein (western blotting) were also examined in paired specimens of HCC and adjacent benign liver. CD151 mRNA was upregulated in HCC tissues compared to paired benign tissues ($p < 0.001$, paired t-test, Fig. 6A). Expression of CD151 was also increased in the paired HCC tissues from the TCGA data (data not shown). Moreover, CD151 protein expression was strongly overexpressed in HCC tissues (Fig. 6B). Finally, using Pearson correlation analysis,

we found a strong inverse correlation between CD151 mRNA and miR-199a-3p expression in HCC (Fig. 6C).

Discussion

Several studies have reported reduced expression of miR-199a in HCC (6-8,22,23). We confirm these results in a new cohort of HCC patients (Fig. 5A) and in the TCGA data set (Fig. 5B). We show that low expression of miR-199a-1 (Fig. 5C) correlates with poor survival in HCC, confirming the data of Fornari *et al* (9). Furthermore, we report that miR-199a-3p regulates CD151. CD151 is a tetraspanin protein family member and has been

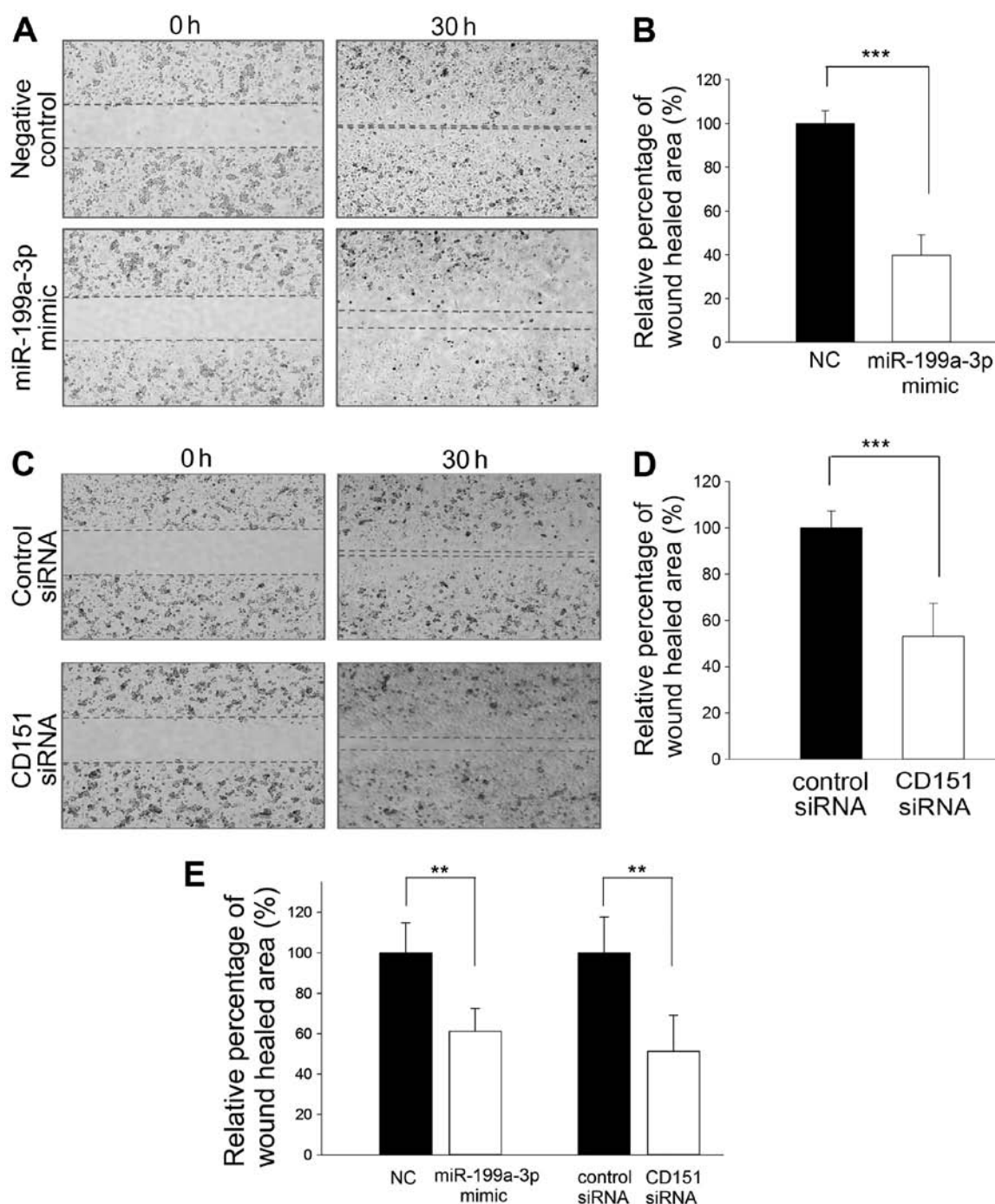


Figure 3. Reintroduction of miR-199a-3p and knockdown of CD151 reduces cell migration *in vitro*. (A and B) Wound healing assays were conducted with negative control and miR-199a-3p mimic transfected SNU-449 cells (4x magnification). (C and D) Wound healing assays were conducted with control siRNA or CD151 siRNA transfected SNU-449 cells (4x magnification). (E) Wound healing assay in SNU-423 cells transfected with miR-199a-3p mimic, siRNA to CD151 or control oligonucleotides. The data are representative of three independent experiments; ** $p < 0.01$, *** $p < 0.001$.

implicated in HCC invasion and migration (12-14). The role of CD151 in HCC invasion and metastasis is believed to rely on its ability to form complexes with laminin-binding integrin receptors ($\alpha 6\beta 1$, $\alpha 6\beta 4$ and $\alpha 3\beta 1$) (14,24) as well as regulate cell-cell and cell-matrix interactions (25). CD151 was previously associated with the mesenchymal phenotype in HCC (14). Knockdown of CD151 in CD151-positive HCCLM3 cells, or overexpression of CD151 in CD151-negative HepG2 cells, altered the mesenchymal phenotype when these cells were cultured along with laminin 5 (14). Induction of EMT

occurred through hyperactivation of the PI3K-Akt-Snail and PTEN feedback pathway (14). Successful knockdown of CD151 did not alter the expression of EMT markers, however our cells were not cultured along with laminin 5 as previously shown (14).

We report that CD151 mRNA and protein are increased in HCC patients. Liver cirrhosis is one predisposing factor to HCC. We compared the expression of miR-199a-3p and CD151 in cirrhotic and non-cirrhotic HCC patients to determine if a relationship exists. Thirty-six percent (%) of the patients in our

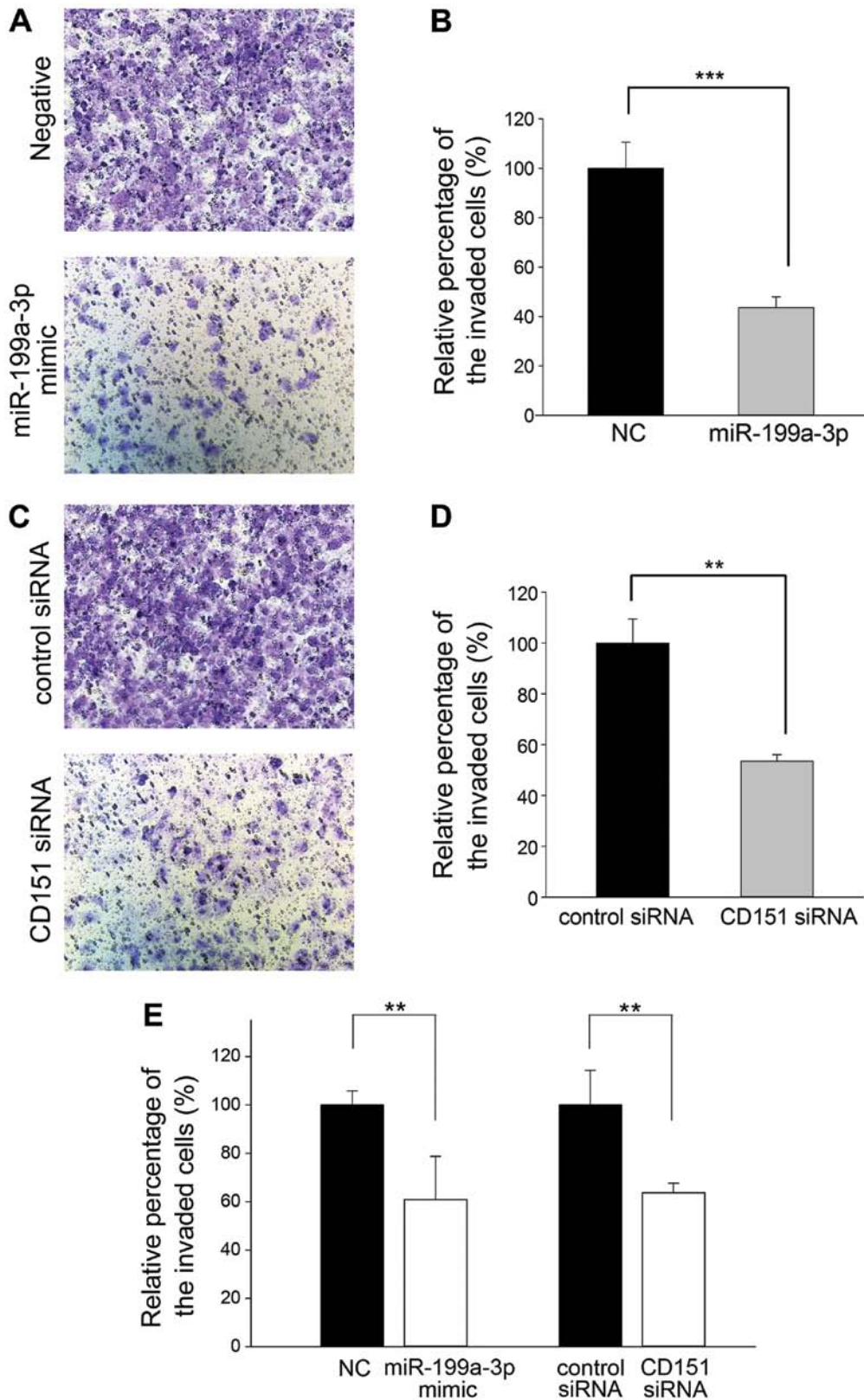


Figure 4. Reintroduction of miR-199a-3p and knockdown of CD151 reduces cell invasion *in vitro*. (A and B) Boyden chamber invasion assays were conducted with negative control and miR-199a-3p mimic transfected SNU-449 cells. (C and D) Boyden chamber invasion assays were conducted with control siRNA or CD151 siRNA transfected SNU-449 cells. (E) Invasion assay in SNU-423 cells transfected with miR-199a-3p mimic, siRNA to CD151 or control oligonucleotides. The number of the invading cells was determined from 5 different fields for each experiment; ** $p < 0.01$, *** $p < 0.001$.

data set had cirrhosis (Table I), however there was no correlation between CD151 or miR-199a-3p and cirrhosis (data not shown). Also no correlation existed between miR-199a-3p and

CD151 in the TCGA data set (data not shown). The regulation of CD151 by miR-199a-3p was further confirmed in HCC patients by the strong negative correlation between these two

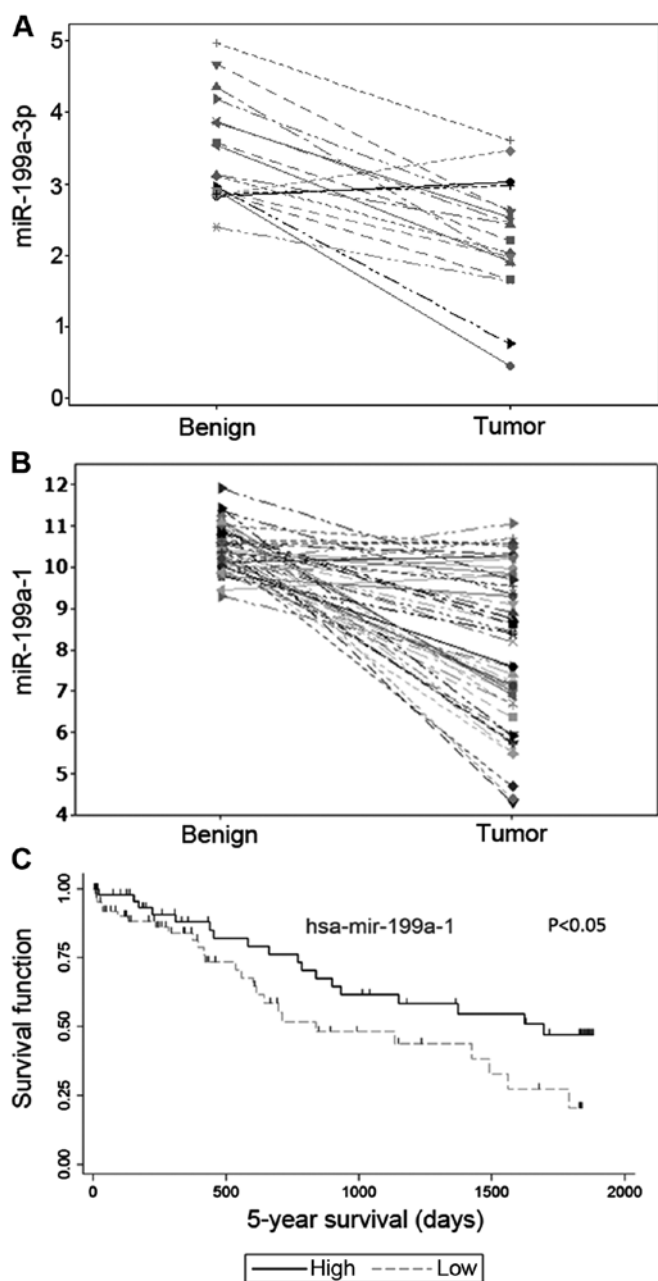


Figure 5. miR-199a-3p is reduced in HCC tissues and low miR-199a-1 expression correlates with poor survival. (A) miR-199a-3p expression relative to 18S rRNA was measured by qRT-PCR in benign and tumor HCC specimens. (B) miR-199a-1 expression in 49 pairs of benign and HCC tissue from TCGA data set. (C) Correlation between miR-199a-1 expression and patient survival. Survival data from 141 patients from the TCGA liver hepatocellular carcinoma were analyzed by the log-rank test.

RNAs (Fig. 6C). While validating additional, putative target genes was beyond the scope of this study, it is of interest to note that miRBase predicts miR-199a-3p to regulate both integrin $\alpha 6$ (ITGA6) as well as integrin $\alpha 3$ (ITGA3). Thus, miR-199a-3p may play a key role in regulating two of the three factors involved in the CD151, integrin $\alpha 3/6$ and laminin 5 interaction, a complex that was reported to be critical for invasion and metastasis in HCC (14).

In addition to CD151, miR-199a-3p has been shown to regulate other tumor and metastasis promoting genes in HCC including c-met (9,10), mTOR (9) and CD44 (11). The reduced

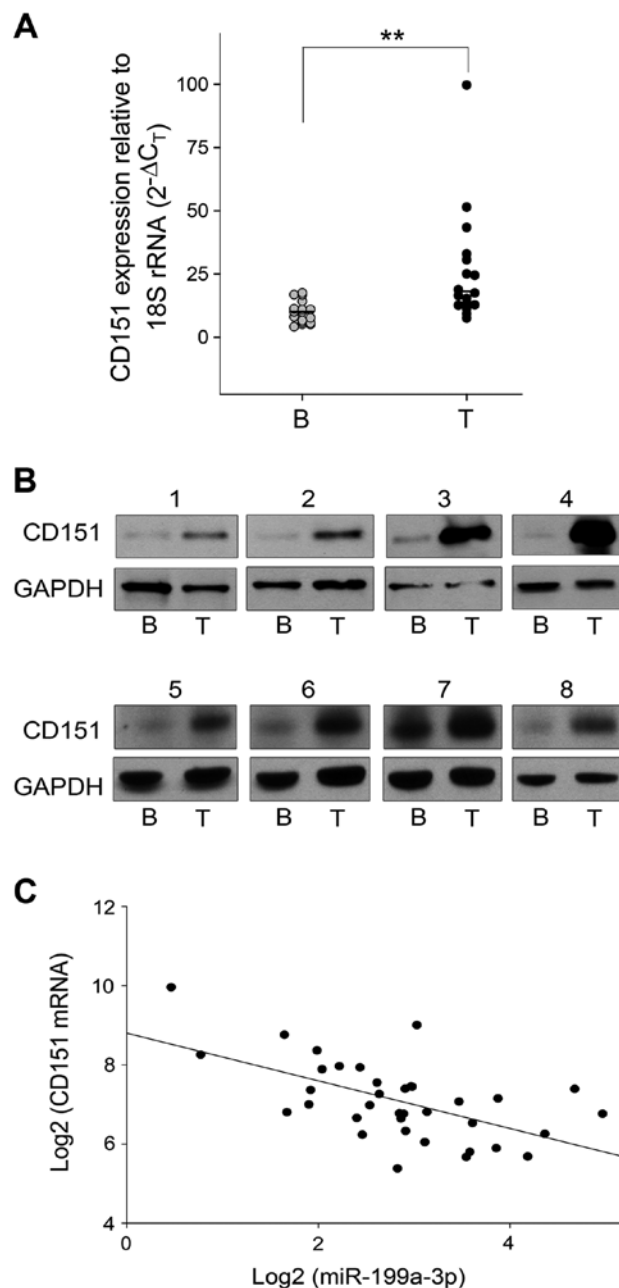


Figure 6. miR-199a-3p and CD151 expression inversely correlates in HCC specimens. (A) Relative expression of CD151 mRNA in benign and HCC specimens was measured by qRT-PCR. (B) CD151 protein expression in HCC and paired adjacent benign tissue was assayed by western blotting. (C) Correlation between miR-199a-3p and CD151 expression as determined by qRT-PCR in HCC specimens by Pearson correlation analysis ($r=-0.58905$, $p<0.001$).

invasion and migration reported cannot be accounted solely by miR-199a-3p suppressing CD151 and the miRNA is likely suppressing known (i.e., c-met, mTOR and CD44) as well as unknown metastasis-related target genes. Since reduced miR-199a-3p expression in HCC would result in increased levels of these cancer promoting genes, it is conceivable that treating advanced HCC using miR-199a-3p oligo mimics could be used as a treatment for a disease that has very few treatment options. Treating HCC with miRNA mimics has great potential as it is well known that oligonucleotides accumulate in highly perfused organs such as the liver (26). In fact,

miR-34 mimic is currently in phase I trial for the treatment of HCC (clinicaltrials.gov).

We previously showed that miR-199a-3p reduced the proliferation of CD44-positive HCC cell but not in CD44-negative HCC cells by reducing CD44 protein levels (11). We report here that miR-199a-3p regulation of CD151 results in decreased invasion and migration, but CD151 does not directly regulate cell growth since siRNA knockdown of CD151 did not affect the proliferation of HCC cells. While CD44 is known to regulate cell adhesion and cell-cell interactions, it is also a well-known marker of stemness. In addition to its role in regulating metastasis-related mRNAs, miR-199a-3p also regulates HCV (27) and HBV replication (28). These findings, coupled with those reported herein, emphasize the critical role that a single deregulated miRNA may have on the cancer phenotype. Reduced miR-199a-3p could influence oncogenesis at various stages of development. Increased HCV and HBV replication may occur early on by reduced miR-199a-3p (28) followed by promoting proliferation in a CD44-dependent manner and increasing invasion and metastasis at later stages.

In conclusion, we showed that CD151 is involved in regulation of *in vitro* invasion and migration but not proliferation of HCC cell lines. miR-199a-3p, a miRNA that is significantly reduced in HCC, directly targets CD151. These data further implicate miR-199a-3p in the progression of HCC and suggests that oligonucleotide therapy using a miR-199a-3p mimic may be effective for treating advanced HCC.

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