

# Metastasin leads to poor prognosis of hepatocellular carcinoma through partly inducing EMT

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**Abstract.** Hepatocellular carcinoma (HCC) is a common malignant cancer worldwide characterized by high metastatic potential and poor prognosis following radical resection. Metastasin is a Ca<sup>2+</sup>-binding protein associated with tumor metastasis. However, the expression and function of metastasin remain unknown. In the present study, we found that the expression of metastasin was upregulated in HCC tissues and positively correlated with poor prognosis following radical resection. Ectopic expression of metastasin *in vitro* induced typical epithelial-mesenchymal transition (EMT) in Hep3B cells including higher capacity of both migration and invasion, increased expression of both Vimentin and N-cadherin and decreased expression of E-cadherin. Knockdown of metastasin produced the opposite results in MHCC97H cells, which indicates that metastasin promotes HCC progression via induction of EMT. SNAIL expression was upregulated by enforced expression of metastasin and, consequently, suppressing upregulation of SNAIL secondary to metastasin overexpression abolished EMT. Collectively, the present results suggest that metastasin leads to HCC EMT partly through upregulating SNAIL and contributes to poor prognosis following radical liver resection.

## Introduction

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer and is the third leading cause of cancer-related mortality worldwide (1). High frequency of intrahepatic and extrahepatic metastasis in the early stage contributes to the poor prognosis of HCC patients, which is also the vital

factor involved in the disappointing survival following curative liver resection (2). Therefore, it is necessary to establish the factors that act as HCC metastatic markers, while playing an important role in the process of metastasis.

Metastasin, also known as S100A4, belongs to the S100 family of Ca<sup>2+</sup>-binding proteins encoded by the *mts1* gene (3,4). Although the relationship between metastasin and tumor metastasis remains unclear, previous investigations in experimental animal models indicated that metastasin is a metastasis factor via enhancing tumor angiogenesis (5), invasion (6) and motility (7). To our knowledge, metastasin expression is diverse in different organs. High metastasin expression has been found in human monocytes, macrophages and polymorphonuclear granulocytes (8), while low level of metastasin protein was detected in the pancreas, colon, thyroid, lung and kidney (9). However, only few investigations have demonstrated the expression and function of metastasin in liver and HCC.

Epithelial mesenchymal transition (EMT) is a program of cancer metastasis which has been characterized as loss of epithelial cell polarity and acquisition of elongated mesenchymal morphology, concomitant with disruption of cell adhesion, increased cell migration, invasion and metastasis (10). In preliminary studies (11-13), EMT was verified to be involved in the cascade of signaling events inducing HCC metastasis (14,15). However, the concrete mechanisms of induction of EMT in HCC remain unclear. Metastasin was found to induce EMT in renal fibrosis and was renamed fibroblast-specific protein (FSP1) (16). In this investigation, we found that metastasin was significantly associated with poor prognosis of HCC and induced EMT through upregulating SNAIL.

## Materials and methods

**Materials.** The rabbit anti-metastasin primary antibody (SC-292281, 1:200 dilution) for western immunoblotting was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The rabbit anti-metastasin primary antibody (ZA-0257) and secondary goat anti-rabbit antibody labeled with biotin (ZDR-5306) for immunohistochemistry was obtained from ZSGB-BIO (Beijing, China). The metastasin-expressing plasmid (RC201616) was from OriGene (Rockville, MD, USA). The human siRNAs targeting both metastasin (SC-106781) and SNAIL (SC-38398) were purchased from Santa Cruz Biotechnology. Other primary antibodies for

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western immunoblotting included rabbit anti-SNAI1 antibody (C15D3, 1:1,000 dilution; Cell Signaling Technology, Danvers, MA, USA), mouse anti-E-cadherin antibody (610181, 1:1,000 dilution; BD Transduction Laboratories, San Jose, CA, USA), rabbit anti-N-cadherin antibody (sc7939, 1:200 dilution; Santa Cruz Biotechnology), and rabbit anti-Vimentin antibody (EPR3776, 1:1,000 dilution; Abcam, Cambridge, MA, USA). Complete Mini Protease Inhibitor Mixture, anti- $\beta$ -actin primary antibody (A-5316) and anti-mouse secondary antibodies conjugated with HRP (A3673) were from Sigma Chemical Co. (St. Louis, MO, USA). The anti-rabbit secondary antibodies conjugated with HRP (ALI3403) were from Biosource Co. (Carlsbad, CA, USA), and ECL reagents were from Amersham/GE Healthcare (Piscataway, NJ, USA).

**Cell culture and transfection.** A previous study showed that there was high metastasin expression in MHCC97H cells, while Hep3B cells expressed metastasin slightly (17). Hence, in the present investigation, MHCC97H cells were selected as the cell model for metastasin knockdown and Hep3B cells were used for metastasin-overexpression study. MHCC97H cell lines were obtained from Fudan University of China and were maintained in our lab. MHCC97H cells, with high metastasis potential frequently used as cell models in the investigation of HCC metastasis, were grown here in DMEM supplemented with 10% FBS. The Hep3B cell line with limited metastasis capacity was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and was cultured in complete MEM medium with 10% FBS.

Both metastasin expressing plasmid and control plasmid (pCMV6-Entry) were transfected into Hep3B cells with Roche FuGENE® 6 Transfection reagent (Indianapolis, IN, USA) and stably expressing clones were selected by G418 at a dose of 300  $\mu$ g/ml for two weeks. Western immunoblotting and qRT-PCR assay confirmed overexpression of metastasin in the selected stably expressing clones (Fig. 1). Metastasin siRNA and scramble siRNA were transfected into MHCC97H cells using the siPORT™ NeoFX™ Transfection Agent from Applied Biosystems (Carlsbad, CA, USA). Knockdown of metastasin was verified by both western immunoblotting and qRT-PCR assay in MHCC97H cells transfected with metastasin siRNA (Fig. 2). Silencing SNAI1 expression of Hep3B cells transfected with metastasin expressing plasmid was carried out with the siPORT™ NeoFX™ Transfection Agent.

**HCC tissue specimens and the matched HCC-adjacent normal tissue specimens.** A total of 41 patients with HCC were enrolled in the study between January 2008 and March 2009, and included 29 men and 12 women (median age, 45 years; range, 34-74 years) who had not received preoperative chemotherapy or embolization. After receiving the routine preoperative examination including chest X-ray, abdominal ultrasonography and computed tomography, all patients underwent liver resection, including radical resection for early HCC and palliative resection for advanced HCC. Tumor tissues and adjacent liver tissues (>2-cm distance to the resection margin) were collected and immediately stored in paraformaldehyde for immunohistochemistry. Clinical data were obtained from the medical records including histopathologic Edmonson classification, clinical tumor-node-metastasis (TNM) grading, maximum

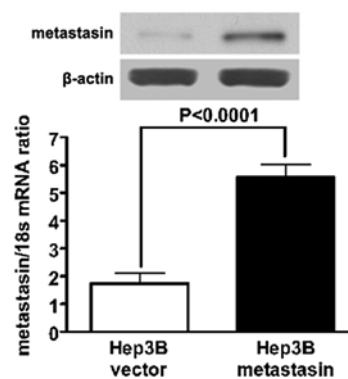


Figure 1. The expression of metastasin was increased after Hep3B cells were stably transfected with metastasin expressing plasmid at the protein and mRNA level.

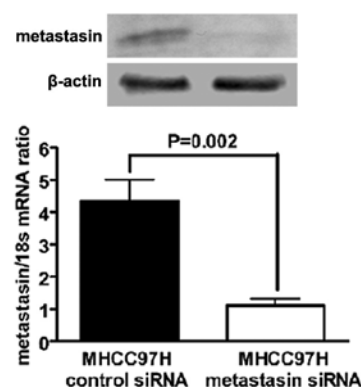


Figure 2. The protein and mRNA expression of metastasin in MHCC97H cells was successfully silenced by siRNA.

tumor diameter, and the tumor-adjacent normal tissues, and were all confirmed by an experienced pathologist who was blinded to clinical information. We also obtained healthy liver tissues from 8 patients without any liver disease. Written informed consent was obtained from all patients. All protocols were approved by the Xi'an Jiaotong University Ethics Committee according to the Helsinki Declaration of 1975.

**Immunohistochemistry staining.** Briefly, all tissues were placed on glass slides, rehydrated and incubated in 3% hydrogen peroxide to block the endogenous peroxidase activity. Following trypsinization, sections were blocked by incubation in 3% bovine serum albumin in PBS. The primary rabbit anti-human metastasin antibody was applied to the slides at a dilution of 1:150 and incubated at 4°C overnight. The tissues were washed three times with PBS and treated with secondary goat anti-rabbit antibody labeled with biotin at 37°C for 30 min. The staining of sections was carried out using HRP-streptavidin conjugates. All sections were visualized with diaminobenzidine and counterstained with hematoxylin. Finally, the sections were dehydrated in alcohol and xylene and mounted onto glass slides.

All slides were detected independently by two experienced pathologists. The staining results were evaluated by an immunohistochemical score combined with the percentage of tumor cells showing specific immunoreactivity. Staining

intensity was classified into four grades: 0 (none), 1 (weak), 2 (moderate) and 3 (strong). The percentage of positive tumor cells was given with the following grades: 0 (<5%), 1 (6-25%), 2 (26-50%), 3 (51-75%) and 4 (>75%). Staining intensity and average percentage of positive tumor cells were assayed in ten independent high-magnification (x400) fields. The total score was calculated by multiplying the staining intensity and the percentage of positive tumor cells. Total score of zero was considered as negative staining. Sections with a total score of 1-4 were defined as the light positive staining, while sections with a score of >4 were considered strongly positive staining.

**Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR).** Total RNA was extracted from HCC cell lines including Hep3B and MHCC97H using the Qiagen RNeasy kit (Valencia, CA, USA). cDNA synthesis was carried out using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) to transcribe 2 µg of total RNA. ABI TaqMan assays were used for qRT-PCR assay in an ABI 7300 system. The ABI TaqMan probes used here were: 18s rRNA (Hs99999901\_s1), metastasin (Hs00243201\_m1), SNAI1 (Hs00195591\_m1), E-cadherin (Hs00170423\_m1), N-cadherin (Hs00983062\_m1) and Vimentin (Hs00185584\_m1). All TaqMan probes were obtained from Applied Biosystems. The mRNA levels of metastasin, SNAI1, E-cadherin, N-cadherin and Vimentin were normalized to 18s rRNA mRNA levels in the same samples. Each measurement was performed 3 times.

**Migration assay.** The wound healing assay was performed as the migration assay. Briefly, HCC cells were seeded onto 6-well plates and cultured to confluency. Scratch wounds were made with a 1,000-µl pipette tip. The wounds were photographed with a phase-contrast microscope at 0, 24 and 48 h. Cell migration was quantitated by measuring the width of the wounds. The experiments were performed with at least six replicates.

**Invasion assay.** A novel quantitative transwell chamber with the Matrigel gel kit - QCM™ 96-well Cell Invasion Assay kit from Millipore (Billerica, MA, USA) was used to detect the invasion capacity of tumor cells, according to the manufacturer's protocol. Briefly,  $\sim 1 \times 10^5$  tumor cells were plated into the upper chamber in serum-free medium and medium with 20% FBS was added into the lower chamber. After 24 h, cells under the invasion chamber were dislodged completely by the cell detachment solution and added into the lysis buffer/dye solution mixture. The results were obtained with a fluorescence plate reader using a 480/520-nm filter set. The experiments were performed with at least six replicates.

**Statistical analysis.** Differences between groups were compared with the Fisher's exact test or the Mann-Whitney test. Differences between the Kaplan-Meier curves of HCC patients with high and low metastasin expression in the HCC tissue in comparison with the adjacent benign tissue were analyzed with the log-rank test.

## Results

*Metastasin is overexpressed in HCC tissues and is associated with poor prognosis.* The majority of positive staining

Table I. Correlation between clinicopathological characteristics and metastasin expression in HCC tissues.

	Metastasin expression		Percentage	P-value
	Positive	Negative		
Gender				
Male	20	9	60	0.514
Female	7	5	58.3	
Age (years)				
≤45	14	8	63.6	0.747
>45	13	6	68.4	
HBV infection				
Yes	18	10	64.3	0.756
No	9	4	69.2	
Liver cirrhosis				
Yes	23	11	67.6	0.594
No	4	3	57.1	
AFP (ng/ml)				
≤400	12	8	60.0	0.440
>400	15	6	71.4	
Intrahepatic metastasis				
Yes	19	5	79.2	0.033
No	8	9	47.1	
Tumor size (cm)				
≤5	14	10	58.3	0.228
>5	13	4	76.5	
Portal vein invasion				
Yes	19	3	86.4	0.001
No	8	11	42.1	
Edmonson classification				
I+II	17	13	56.7	0.040
III+IV	10	1	90.9	
TNM staging				
I+II	12	11	52.2	0.037
III+IV	15	3	83.3	

cells showed diffuse cytoplasmic staining of metastasin, while nuclear staining was found in a few cells (Fig. 3A). In 41 pairs of HCC tissues and adjacent liver tissues, there were 27 (65.9%) positive metastasin staining HCC tissues compared to 8 (19.5%) positive staining adjacent liver tissues (Fig. 3A and B). The staining of metastasin in the 8 healthy liver tissues was negative (Fig. 3C). These results clearly indicate that metastasin was overexpressed in HCC tissues ( $P < 0.001$ ). After analyzing the relationship between metastasin expression in HCC tissues and clinicopathological characteristics (Table I), we found that positive metastasin expression was significantly related to intrahepatic metastases ( $P = 0.033$ ), portal vein invasion ( $P = 0.001$ ), advanced TNM staging ( $P = 0.037$ ) and high Edmonson classification ( $P = 0.04$ ), suggesting that metastasin expression in HCC tissues contributes to HCC metastasis.

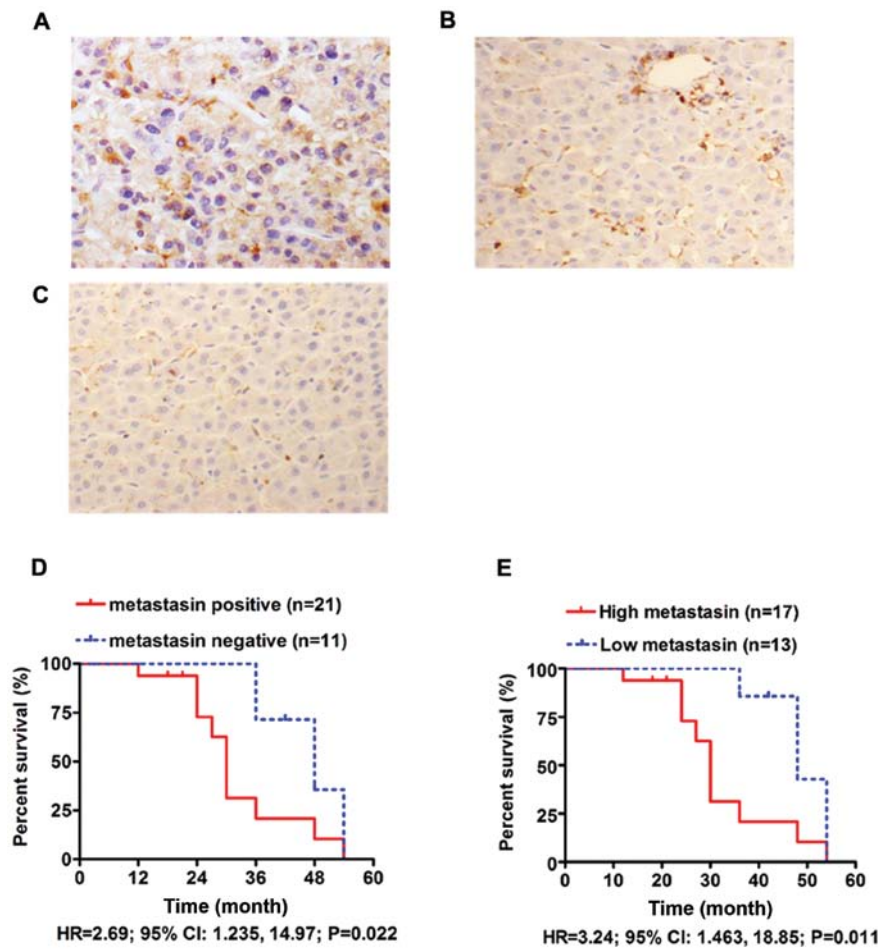


Figure 3. Metastasin is frequently overexpressed in HCC tissues compared to adjacent normal tissues and is associated with poor survival following liver resection. (A) Immunohistochemistry staining of metastasin in HCC tissue. (B) Immunohistochemistry staining of metastasin in tumor-adjacent normal liver tissue. (C) Immunohistochemistry staining of metastasin in normal liver tissue. (D) Differences between the Kaplan-Meier curves of HCC patients in the metastasin positive group and those in the metastasin negative group. (E) Differences between the Kaplan-Meier curves of HCC patients in the high metastasin group and those in the low metastasin group. Original magnification of immunohistochemistry, x200.

We obtained follow-up information from 32 of the 41 HCC cases (78%). The median duration of follow-up was 24 months. The 3-year survival rate of the metastasin positive group was 20.8% compared to 71.4% in the metastasin negative group. As compared by the Kaplan-Meier survival curve, HCCs with metastasin expression were found to have poorer survival (HR=2.69; 95% CI: 1.235, 14.97; P=0.022) (Fig. 3D). We also compared the survival curves between the high metastasin group, in which HCC tissues expressed more metastasin than adjacent normal tissues, and in the low metastasin group. The 3-year survival rate of the high metastasin group was found to be 20.8% compared to 85.7% in the low metastasin group. The high metastasin group was found to have clearly worse overall survival than the low metastasin group (HR=3.24; 95% CI: 1.463, 18.85; P=0.011) (Fig. 3E). These data showed that metastasin expression in HCC tissues was markedly associated with poor prognosis.

*Ectopic expression of metastasin in Hep3B cells promotes cell migration and invasion, and induces EMT.* Ectopic expression of metastasin was confirmed by both qRT-PCR and western immunoblotting (Fig. 1). To investigate the effect of metastasin on HCC migration, wound healing assay was carried out and

showed that the migration rate of Hep3B cells stably transfected with metastasin expressing plasmid (Hep3B metastasin cells) was significantly faster than that of Hep3B cells transfected with vector plasmid (Hep3B vector cells) at both 24 and 48 h after scratching (both P-values were 0.002; Fig. 4A). Similar results were obtained from the quantitative invasion assay, which showed that the invasion ability of Hep3B cells was greatly increased by ectopic expression of metastasin (P=0.001; Fig. 4B).

To investigate whether metastasin promotes migration and invasion by inducing EMT, we examined expression of EMT markers of Hep3B cells following overexpression of metastasin. SNAIL1, a well-known EMT inducer, was found to be upregulated at both the mRNA and the protein level. qRT-PCR assay revealed that E-cadherin mRNA expression of Hep3B cells was decreased by ~60% by ectopic expression of metastasin (Fig. 4C). The protein expression of E-cadherin was verified to be suppressed in Hep3B metastasin cells by western immunoblotting (Fig. 4D). On the other hand, as shown in Fig. 4D, the expression of both N-cadherin and Vimentin, which are considered mesenchymal markers, was found enhanced at the mRNA and protein levels by metastasin overexpression. Collectively, the data presented here indicate

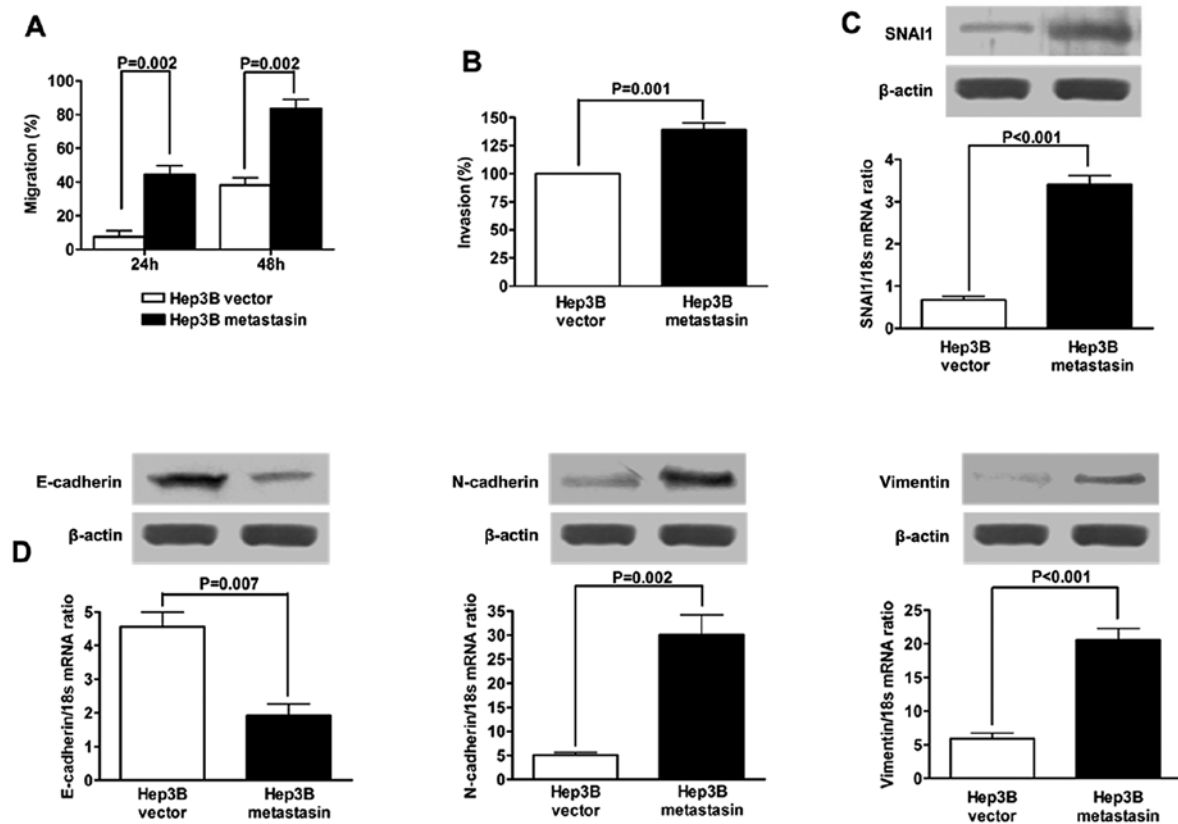


Figure 4. Forced expression of metastasin promotes both migration and invasion and induces EMT phenotype of Hep3B cells. (A) Migration of Hep3B cells was significantly increased by metastasin overexpression as assessed by the wound healing assay. (B) Forced expression of metastasin clearly enhanced the invasion capacity of Hep3B cells. (C) SNAI1 expression of Hep3B cells was found upregulated >3-fold following metastasin overexpression by both qRT-PCR and western immunoblotting. (D) qRT-PCR and western immunoblotting showed that ectopic expression of metastasin decreased E-cadherin expression and markedly increased the expression of two types of mesenchymal markers, N-cadherin and Vimentin.

that metastasin promotes migration and invasion of Hep3B cells via induction of EMT.

**Knockdown of metastasin in MHCC97H cells suppresses cell migration and invasion and reverses EMT.** To further confirm the observation regarding metastasin overexpression, we silenced metastasin expression of MHCC97H cells by transfection of metastasin siRNA (Fig. 2). As shown in Fig. 5A, wound healing assay showed that knockdown of metastasin significantly suppressed mobility of MHCC97H cells (24 h after scratching,  $P=0.032$ ; 48 h after scratching,  $P=0.011$ ). Accordingly, the invasion ability of MHCC97H cells was markedly attenuated by silencing metastasin ( $P=0.002$ ; Fig. 5B).

Next, we tested if knockdown of metastasin downregulated SNAI1 expression and reverted EMT in MHCC97H cells. SNAI1 expression was significantly decreased by silencing metastasin at both the protein and the mRNA level, as assessed by western immunoblotting and qRT-PCR, respectively (Fig. 5C). Further examination for the expression of EMT markers showed that knockdown of metastasin promoted E-cadherin expression and suppressed the expression of N-cadherin and Vimentin (Fig. 5D). The complete opposite results obtained here further confirm the hypothesis that metastasin induces EMT in HCC cells and then promotes cell migration and invasion.

#### Suppression of SNAI1 blocks EMT induced by metastasin.

To investigate the role of SNAI1 in metastasin-induced EMT, we silenced SNAI1 upregulated by metastasin overexpression and re-evaluated EMT phenotype of Hep3B metastasin cells. qRT-PCR and western immunoblotting confirmed that SNAI1 expression of Hep3B cells was knocked down effectively by siRNA (Fig. 6A). E-cadherin expression was increased significantly, while the expression of both N-cadherin and Vimentin were decreased (Fig. 6B). The migration and invasion of Hep3B metastasin cells were both significantly inhibited (Fig. 6C and D). These data showed that EMT phenotype of Hep3B cells induced by metastasin was reversed following SNAI1 knockdown.

#### Discussion

HCC is one of the most common malignant tumors worldwide with increasing incidence rates. Radical hepatic resection at the early stage is the most important and common curative treatment for HCC. However, due to the lack of typical symptoms at the early stage and high metastatic capacity, the majority of HCC patients at present have no opportunity to receive the curative resection. To date, metastasis of HCC remains largely incurable due to its systemic characteristics and the resistance to the existing therapeutic agents. Therefore, it is critical to explore the mechanism underlying HCC metas-

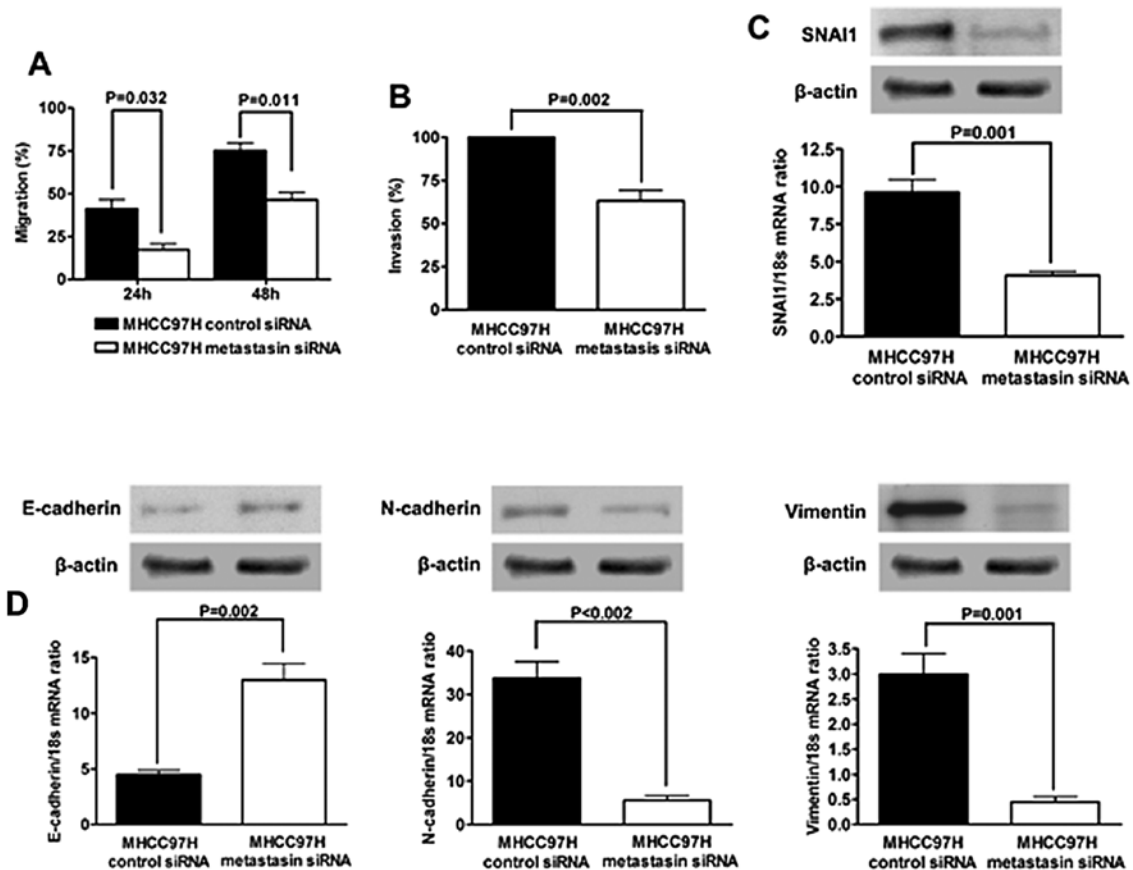


Figure 5. Knockdown of metastasin inhibits both migration and invasion ability and reverses EMT phenotype of MHCC97H cells. (A) Migration of Hep3B cells was found significantly suppressed by silencing metastasin, as assessed by the wound healing assay. (B) Knockdown of metastasin clearly inhibited the invasion potential of MHCC97H cells. (C) SNAI1 expression of MHCC97H cells was found evidently decreased by knockdown of metastasin by both qRT-PCR and western immunoblotting. (D) Silencing metastasin increased E-cadherin expression and downregulated the expression of N-cadherin and Vimentin significantly at both the mRNA and the protein level.

tasis and to develop effective clinical prognostic biomarkers and therapeutic agents.

The metastasin protein encoded by the human *mtsl* gene located at position 1q21 on chromosome 1 is a type of polypeptide of 101 amino acids with the molecular mass of 11.5 kDa. Previous studies revealed that the transcription of metastasin is controlled by regulatory elements in its first intron via binding with several transcription factors, such as I- $\kappa$ B (18,19). Epigenetic investigations also found metastasin transcription was strongly affected by the methylation of both its enhancer and silencer elements, which could contribute to its aberrant expression in diverse types of human cancer (20-22). Clinical research on breast cancer patients with regional invasion but operable stage I and II, reported by Rudland *et al* (23), showed that during the 19-year follow-up period, the median survival of the metastasin negative group was 228 months vs. 47 months in the metastasin positive group. A similar result was reported by Lee *et al* (24) showing that metastasin was related with poor outcome of T1N0M0 breast cancer and could be a biomarker of early metastasis of breast cancer. Aside from the investigations on breast cancer, numerous studies of different types of human cancer, including colon cancer (25), gastric cancer (26,27), pancreatic ductal carcinoma (28), renal carcinoma (29), found that metastasin expression was significantly overexpressed in malignant tissues and was associated

with poor survival. To our knowledge, there are few reports about the expression and function of metastasin on HCC. In this study, we found metastasin was aberrantly upregulated in HCC tissues and was associated with poor outcome following liver resection, which indicates metastasin could be a potential predictive marker and therapy target for HCC.

In the present study we initially investigated the mechanism by which metastasin leads to poor survival of HCC. In general, metastasin has no enzymatic activity and works via binding and interacting with other proteins, including p53, P37, S100A1, CCN3 (30,31). Numerous *in vivo* experiments showed that metastasin promotes tumor metastasis in mouse models. Ambartsumian *et al* (32) verified that metastasin leads to higher metastatic capacity of mammary tumor via transgenic mouse overexpressing metastasin in mammary epithelium. The knockdown *in vivo* experiments on lung carcinoma and osteosarcoma also showed silencing metastasin suppressed the metastatic potential of tumor cells (33,34). Although several investigations showed overexpression of metastasin induced downregulation of E-cadherin and upregulation of MMPs (6,35,36), the underlying mechanism by which metastasin promotes tumor metastasis has yet to be fully elucidated. Here, we found ectopic expression of metastasin in Hep3B cells increased cell mobility and invasion. SNAI1, a renowned EMT inducer, was also found

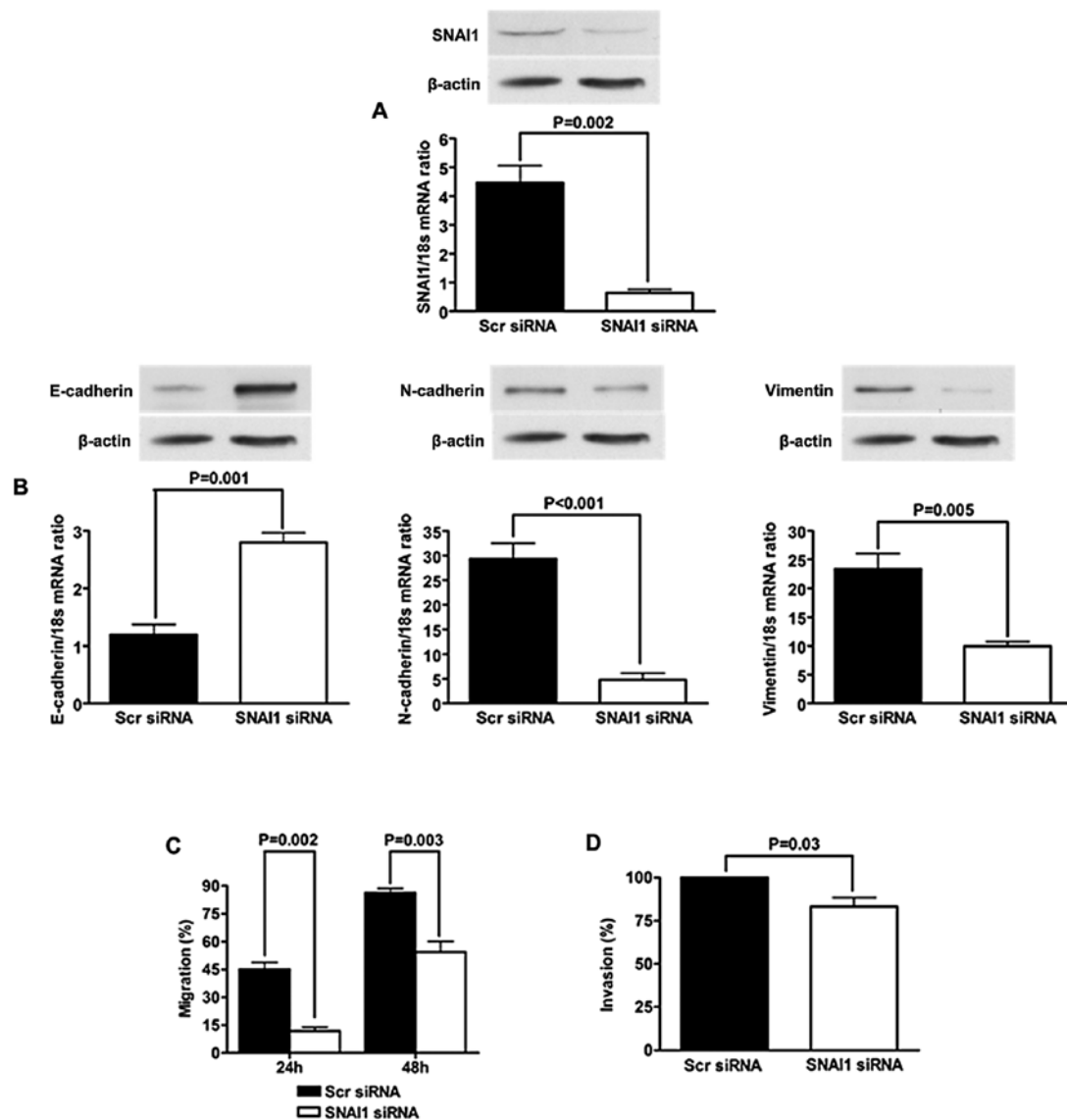


Figure 6. Suppression of SNAIL blocks EMT induced by metastasin. (A) SNAIL upregulation by overexpression of metastasin was abolished entirely by transfecting SNAIL siRNA into Hep3B cells one day prior to transfecting metastasin expressing plasmid. (B) Suppression of SNAIL clearly increased E-cadherin expression and decreased the expression of both N-cadherin and Vimentin in Hep3B metastasin cells, as assessed by qRT-PCR and western immunoblotting. (C) Cell migration of Hep3B metastasin cells was significantly inhibited by suppression of SNAIL, as assessed by the wound healing assay. (D) The invasion capacity of Hep3B metastasin cells was inhibited by suppression of SNAIL.

upregulated by metastasin overexpression, suggesting that metastasin induced EMT in Hep3B cells. Further examination showing E-cadherin was downregulated and both N-cadherin and Vimentin were upregulated confirms the EMT phenotype of Hep3B cells induced by ectopic expression of metastasin. Conversely, knockdown of metastasin in MHCC97H cells led to the opposite results, which further verifies that metastasin induces EMT phenotype of HCC cells through upregulating SNAIL. Although metastasin was found to enhance SNAIL expression in both Hep3B and MHCC97H cells, there is no evidence to support that metastasin could regulate the transcription of SNAIL directly. Our group sought to find the metastasin-binding site of SNAIL promoter by carrying out EMSA (unpublished data) and did not get any positive results, which indicates that metastasin could upregulate SNAIL in indirect ways. To clarify the role of SNAIL upregulated on metastasin-driven EMT, we successfully inhibited

the upregulation of SNAIL after stably overexpressing metastasin in Hep3B cells by SNAIL siRNA, and found E-cadherin expression was increased and the expression of both N-cadherin and Vimentin were clearly decreased. Consistently, both cell mobility and invasion were inhibited significantly. Consequently, these data indicate EMT induced by metastasin is reversed by suppressing SNAIL in Hep3B cells and SNAIL is involved in the intracellular signal cascade of metastasin-induced EMT.

In summary, this investigation shows metastasin is over-expressed in HCC and is markedly related with poor survival. Metastasin induces EMT phenotype of HCC via upregulating SNAIL, which could explain initially how metastasin leads to poor prognosis of HCC. Therefore, metastasin is a potential clinical biomarker and target for anti-HCC therapy, and further investigation is required to elucidate the concrete mechanism by which metastasin promotes HCC progression.

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