SIN1 Promotes Invasion and Metastasis of Hepatocellular Carcinoma by Facilitating Epithelial-Mesenchymal Transition

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BACKGROUND: Stress-activated protein kinase (SAPK) interacting protein 1 (SIN1) is essential for early embryonic development and is the key regulator of Akt, which plays an important role in various pathological conditions such as cancer. However, the biological function and clinical significance of SIN1 in hepatocellular carcinoma (HCC) remains unknown. METHODS: Real-time quantitative reverse-transcriptase polymerase chain reaction analysis, western blot analysis, and immunohistochemical staining were used to test the expression level of SIN1, and its correlation with clinicopathologic parameters as well as the prognosis for patients with HCC were analyzed. In addition, the biological function and molecular mechanisms of SIN1 in HCC were investigated. RESULTS: SIN1 levels were elevated predominantly in HCC tissues, and its level in solitary large HCC was significantly lower than those in nodular HCC (P=.016), but showed no significant differences between solitary large HCC and small HCC (P>.05). Levels of SIN1 were up-regulated in highly metastatic HCC cell lines (HCCLM3 and MHCC97-H), whereas their invasion and migration significantly decreased after depletion of SIN1. High expression of SIN1 was associated with tumor number (P=.012), capsular formation (P=.037), and venous invasion (P=.023) and was an independent risk factor for overall survival (P=.046). Finally, SIN1 was capable of promoting invasion and metastasis of HCC by facilitating epithelial-mesenchymal transition. CONCLUSIONS: The current findings suggest that SIN1 plays an important role in HCC invasion and metastasis by facilitating epithelial-mesenchymal transition. Cancer 2013;119:2247-57. © 2013 American Cancer Society.

KEYWORDS: SAPK interacting protein 1; hepatocellular carcinoma; invasion; metastasis; epithelial-mesenchymal transition.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common cancers and ranks as the second leading cause of cancer-related death in men worldwide.1,2 The overall survival of patients with HCC remains unsatisfactory because of a high incidence of recurrence and metastasis.3,4 In recent decades, various molecules have been reported to play a role in invasion and migration of HCC, such as RhoC, Egfl7, FBI-1, and HSF1.5-8 Although these findings represent significant progress in the field, the mechanisms underlying HCC metastasis are still largely unknown. Therefore, it is very important to identify the effective biomarkers to predict outcome and prevent postoperative recurrence and metastasis of HCC.

Previous studies showed that SAPK interacting protein 1 (SIN1) is essential for early embryonic development and is the key regulator of Akt which play an important role in various pathological conditions such as cancer.9-11 SIN1 was identified as a key TORC2 component and regulator of the Akt pathway that positively controls Akt-Ser473 phosphorylation and activation. However, the biological function and clinical significance of SIN1 in HCC remains unknown.

Therefore, we carried out the current study to explore the potential role of SIN1 in HCC by determining its expression in human HCC tissues as well as cell lines. Furthermore, the biological function and the underlying molecular mechanisms of SIN1 in HCC invasion and metastasis were also investigated.

MATERIALS AND METHODS

Patients and Tissue Specimens

A total of 60 HCC specimens were obtained from patients who underwent hepatectomy at the Department of Surgery, Xiangya Hospital of Central South University, Hunan, China, from January 2003 to December 2010. None of the patients in our study received neoadjuvant chemotherapy. These patients included 49 males and 11 females with median
age of 45 years (range, 19-68). Among these patients, 25 matched fresh HCC specimens and adjacent nontumorous liver tissue (ANLT) were selectively employed for real-time quantitative reverse-transcription polymerase chain reaction (qRT-PCR) and western blot analysis. The diagnosis for each patient was confirmed by histopathology. Prior informed consent was obtained, and the study protocol was approved by the Ethics Committee of Xiangya Hospital.

qRT-PCR
Total RNA was extracted from cell lines or frozen tumor specimens using Trizol reagent (Invitrogen, Carlsbad, Calif) according to the instructions. Real-time PCR was performed using the SYBR Green Real-time PCR Master Mix (Toyobo, Osaka, Japan) as described. The primers of SIN1 were as follows: forward, 5'-TCCACA-GACTGCGATTCAACAC-3', reverse, 5'-TCTTCAGCAAGGTACAGGCACA-3', GAPDH was used as a control using the following primers: forward, 5'-GCACCGTCAAGGCTGAGAAC-3', reverse, 5'-TGGTGAAGACGCCAGTGGA-3'. The results were analyzed using the 2^-DeltaDeltaCT method with the formula: 

\[ \Delta \Delta CT = (Ct_{HCC} - Ct_{GAPDH}) - (Ct_{ANLT} - Ct_{GAPDH}) \]

Western Blot Analysis
Total proteins were extracted and separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto polyvinylidene fluoride membranes (Millipore, Bedford, Mass). The blotted membranes were incubated anti-human SIN1 antibody (Abcam, Cambridge, Mass), anti-human p-Akt antibody (Santa Cruz Biotechnology, Santa Cruz, Calif), anti-human Akt antibody (Santa Cruz Biotechnology), anti-human p-P70S6K antibody (Santa Cruz Biotechnology), anti-human b-catenin antibody (Santa Cruz Biotechnology), anti-human Vimentin antibody (Santa Cruz Biotechnology), anti-human Snail antibody (Santa Cruz Biotechnology), anti-human E-cadherin antibody (Santa Cruz Biotechnology), anti-human N-cadherin antibody (Santa Cruz Biotechnology), or anti-human matrix metalloproteinase 9 (MMP9) antibody (Santa Cruz Biotechnology), and then an appropriate secondary antibody (Santa Cruz Biotechnology). b-Actin protein determined by its antibody (Santa Cruz Biotechnology) was used as a loading control.

Immunohistochemistry
Formalin-fixed paraffin sections were stained for SIN1, Snail, Vimentin, MMP9, E-cadherin, and N-cadherin using the streptavidin-peroxidase system (Zhong-shan Goldenbridge Biotechnology, Beijing, China). Negative control slides were probed with goat serum followed by the secondary antibody under the same conditions. The expression levels of SIN1 were scored using a 4-point scale according to the percentage of positive hepatocytes: 0, ≤ 10% positive; 1+, 11% to 25% positive; 2+, 26% to 50% positive; 3+, ≥51% positive. The protein expression of SIN1 was thus considered negative if scored 0, and 1+, 2+, and 3+ were considered positive. SIN1 expression in HCC specimens was also divided into a low-expression group (0 or 1+) and a high-expression group (2+ or 3+).

Follow-Up and Prognostic Study
All patients were regularly followed up by the same surgical team in our hospital, with surveillance for the recurrence and metastasis by clinical examination, alpha-fetoprotein levels, and ultrasonography or computed tomography scan every 3 months. The follow-up period was defined as the interval between the date of operation and the date of patient death or last follow-up. Deaths from other causes were treated as censored cases. Eight conventional variables together with SIN1 expression were tested in all 60 patients: age, sex, liver cirrhosis, capsular formation, tumor size, tumor number, Edmondson-Steiner grade, and venous invasion.

Immunofluorescence
F-actin immunofluorescence staining was used to analyze the cell skeleton. Cells grown on cover slides were fixed and then incubated with rhodamine-conjugated phalloidin (Beyotime Institute of Biotechnology, Jiangsu, China). F-actin filaments were observed and analyzed with a Nikon 80i fluorescence microscope (Nikon, Tokyo, Japan).

Cell Lines and Cell Culture
HepG2 cell lines were purchased from the ATCC (Manassas, Va). LO2, SMMC-7721, MHCC97-L, MHCC97-H, and HCCLM3 cell lines were purchased from the Liver Cancer Institute of Fudan University. All cell lines were routinely cultured in Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum and antibiotics at 37°C with 5% CO2.

Plasmid Construction and Infection
The plasmid carrying following small hairpin RNAs (shRNA) designed to knock down SIN1 messenger RNA (mRNA) were purchased from GeneChem (Shanghai, China). The 3 candidate hairpin sequences were:
sequence-1: sense, 5’-GATCCCTTAAGCAATCAGCACTAAACTGAGTTTATAGCTCGATGTAGGCTTATGAGGATTTGGAAT-3’, antisense, 5’-AGCTATCCAAAAACTAAGCAATCAGCACTAAACTGAGTTTATAGCTCGATGTAGGCTTATGAGGATTTGGAAT-3’; sequence-2: sense, 5’-GATCCCTTAAGCAATCAGCACTAAACTGAGTTTATAGCTCGATGTAGGCTTATGAGGATTTGGAAT-3’, antisense, 5’-AGCTATCCAAAAACTAAGCAATCAGCACTAAACTGAGTTTATAGCTCGATGTAGGCTTATGAGGATTTGGAAT-3’; sequence-3: sense, 5’-GATCCCTTAAGCAATCAGCACTAAACTGAGTTTATAGCTCGATGTAGGCTTATGAGGATTTGGAAT-3’, antisense, 5’-AGCTATCCAAAAACTAAGCAATCAGCACTAAACTGAGTTTATAGCTCGATGTAGGCTTATGAGGATTTGGAAT-3’. HCCLM3 and MHCC97-H cells were transfected with the shRNA plasmid using Lipofectamine 2000 (Invitrogen, Carlsbad, Calif) according to the manufacturer’s instructions.

Wound Healing and Transwell Assay
For the wound healing assay, cells were seeded into 35-mm dishes precoated with fibronectin. When cells reached 100% confluence, a scratch was made with a pipette tip. The cells were then cultured for another 48 hours and a micrograph was taken every 24 hours for the Transwell assay, approximately 1 × 10^5 cells were placed into the upper chamber of the insert with Matrigel (BD Biosciences, Woburn, Mass). The cells were cultured in serum-free medium in 5% CO₂ for 48 hours, then cells in the upper chamber were removed with cotton swabs and stained with a solution containing 0.1% crystal violet and 20% methanol. The number of cells that adhered to the lower membrane of the inserts was counted. For each experimental group, the invasion assay was performed in triplicate, and 3 random fields were chosen for cell number quantification.

HCC Metastatic Mouse Model
The HCC metastatic mouse model was constructed by using HCCLM3NC and HCCLM3SIN1-RNAi cells. Cells (5 × 10^5) of different groups were injected subcutaneously into the left upper flank regions of nude mouse (4 weeks of age, male, BALB/c). After 1 month, the subcutaneous tumor tissues were removed and implanted into the liver of nude mice (4 in each group). After 6 weeks, the livers of mice were fixed with phosphate-buffered neutral formalin, sectioned serially, and stained with hematoxylin and eosin for standard histologic examination. The mice were housed and manipulated according to the protocols approved by the Medical Experimental Animal Care Commission.

STATISTICAL ANALYSIS
All data were analyzed using the statistical software SPSS, version 17.0 for Windows (SPSS, Chicago, Ill). The Fisher’s exact test was used for statistical analysis of categorical data, whereas independent t tests were used for continuous data. Survival curves were constructed using the Kaplan-Meier method and evaluated using the log-rank test. The Cox proportional hazards regression model was established to identify factors which were independently associated with the overall survival of HCC patients. P values < .05 was considered statistically significant.

RESULTS
SIN1 Expression Was Significantly Elevated in Human HCC Tissues
The expression of SIN1 mRNA was highly elevated in HCC tissues compared with ANLTs, and the median fold-change was 2.2 (range, 1.0-20.1). Increased SIN1 mRNA was also evidenced in thrombus than that in ANLTs, with the median fold-change of 4.02 (range, 2.9-5.1) (Fig. 1A). Moreover, the results of semiquantitative RT-PCR, showing the significantly elevated SIN1 mRNA in HCC tissues than ANLTs (Fig. 1A), also confirmed these results. Consistent with mRNA expression, the western blot results showed that the expression of SIN1 protein in HCC tissues was also significantly higher than that in the corresponding ANLTs (0.80 ± 0.11 versus 0.22 ± 0.07; P < .01) (Fig. 1B). The SIN1 protein levels in ANLTs were higher than those in normal liver tissues; however, the difference was not statistically significant (Fig. 1B, P > .05).

Association Between SIN1 Expression and Metastatic Potential of HCC
We compared SIN1 expression in 3 special types of HCCs: solitary large hepatocellular carcinoma (SLHCC), small hepatocellular carcinoma (SHCC), and nodular hepatocellular carcinoma (NHCC). The results showed that the SIN1 mRNA level in SLHCC was lower than those in NHCC. Both qRT-PCR and western blot showed the significantly lower levels of SIN1 in SLHCC than those in NHCC, but no significant differences between levels of SIN1 in SLHCC and SHCC were found (Fig. 1C, P > .05). Furthermore, we examined the SIN1 expression in 6 liver cell lines, including a normal liver cell line (L02 cells), and 5 HCC cell lines with different metastatic potential (HepG2, MHCC97-L, MHCC97-H, SMMC-7721, and HCCLM3).13-15 Either real-time qRT-PCR or western blot showed that the SIN1 expression levels were significantly lower in SLHCC than those in NHCC.
significantly higher in HCC cell lines than those in normal liver cells (LO2) (Fig. 1D,E). We also compared the expression of SIN1 in 4 HCC cell lines (HepG2, MHCC97-L, MHCC97-H, and HCCLM3) and found that HCCLM3 and MHCC97-H with strongest metastatic abilities had the highest mRNA and protein expression levels of SIN1, followed by MHCC97-L, and then HepG2 cells (Fig. 1D,E).

Figure 1. Overexpression of SAPK interacting protein 1 (SIN1) in hepatocellular carcinoma (HCC) tissues and cells. (A) The real-time quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) and RT-PCR results show that the expression level of SIN1 messenger RNA (mRNA) in HCC tissues was highly elevated than that in adjacent nontumorous liver tissue (ANLT). The median fold-change was 2.2. The expression level in thrombus was highly elevated than that in HCC tissues. The median fold-change was 4.02. The expression of SIN1 mRNA in ANLTs was used to normalize (value set to 1) the expression of SIN1 mRNA in HCC tissues and in thrombus (T). (B) The representative western blot results showed that SIN1 protein in HCC tissues were significantly higher than those in ANLTs and normal liver (NL). Student t test shows the expression levels of SIN1 protein in HCC tissues are significantly higher than those in ANLTs (P < .01), but there were not significant differences between ANLTs and normal liver tissues (P > .05). (C) The real-time qRT-PCR showed that the SIN1 mRNA level in SLHCC was lower than those in NHCC. Western blot results showed that SIN1 protein level in SLHCC was significantly lower than those in NHCC (P = .016), but showed no significant differences between SLHCC and SHCC (P > .05). (D,E) SIN1 mRNA and protein expression levels were detected by real-time qRT-PCR and western blot in LO2 cells and 5 HCC cell lines. The relative expression fold in HepG2, MHCC97-L, MHCC97-H, SMMC-7721, and HCCLM3 was 2.01, 2.18, 3.09, 4.32, and 4.51, respectively. Western blot results showed that MHCC97-H cells had the highest SIN1 expression level compared to LO2 cells (P < .01), followed by HCCLM3. (F) Lipofectamine 2000 was used to transfected shRNA for Sequence 1, Sequence 2, Sequence 3, and Sequence c. The western blot results showed that the Sequence 2 is much more effective than others.

Abbreviation: bp, base pair; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
Correlations of SIN1 Expression With Clinicopathologic Characteristics and Prognosis of HCC

Immunohistochemistry showed that SIN1 expression was significantly higher in HCC than in ANLTs (Fig. 2). We also found that SIN1 expression negatively correlates with capsular formation (P = .037) and positively correlates with vascular invasion (P = .023), tumor numbers (P = .012), and TNM stage of HCC (P = .020) (Table 1).

According to the immunohistochemistry results, all patients with HCC were divided into 2 groups: high expression group in which SIN1 expression scored as 2+ or 3+ (n = 39); low expression group in which SIN1 expression scored as 1+ or negative (n = 21). The high-expression group had lower overall survival rates and disease-free survival rates than those of the low-expression group (P = .012 and P = .001, respectively) (Fig. 2). In addition, results from the multivariate Cox regression analysis indicate that high SIN1 expression (relative risk, 1.373; P = .046) together with vascular invasion (relative risk, 2.536; P = .038), tumor numbers (relative risk, 1.687; P = .048), and TNM stage (relative risk, 2.236; P = .037) were independent prognostic factors for overall survival of HCC patients (Table 2).

**Suppression of SIN1 Expression by shRNA**

To determine whether SIN1 contributes to the progression of HCC, we employed shRNA to inhibit the expression of SIN1 in HCC cells (Fig. 1F). Western blot was then performed to assess the efficiency of SIN1 knockdown in HCC cells. Sequence 2 reduced the level of SIN1 by more than 90% (Fig. 1F). The other 2 sequences, candidates 1 and 3, reduced the level of SIN1 by 60%. HCCLM3 and MHCC97-H cells transfected with sequence 2 and the scramble sequence were designated, for convenience, HCCLM3SIN1-RNAi and HCCLM3NC; MHCC97-HSIN1-RNAi and MHCC97-HNC, respectively.

**SIN1 Promotes Invasion and Metastasis of HCC Cells**

We employed the wound healing and Transwell assays to analyze the function of SIN1 in HCC cell invasion and migration (Fig. 3A-D). The wound healing assay showed that HCCLM3SIN1-RNAi cells closed much slower than that of HCCLM3NC cells (45% versus 95%, P < .01). Meanwhile, the closure of MHCC97-HSIN1-RNAi was also significantly slower than that of MHCC97-HNC (49% versus 97%, P < .01), suggesting a role for SIN1 in the regulation of HCC cell migration. To further confirm the results, we performed a transwell assay and the results showed that the numbers of HCCLM3 SIN-RNAi cells which passed through the Matrigel was significantly less than that of HCCLM3NC cells (56 ± 13 versus 161 ± 18, P < .01). The results obtained from MHCC97-HSIN1-RNAi cells and MHCC97-HNC, respectively.

**SIN1 Regulates the Cell Skeleton**

F-actin immunofluorescence staining was used to analyze the cell skeleton (Fig. 3E). The results showed that the

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**TABLE 1. Correlations Between SAPK Interacting Protein 1 (SIN1) Expression and Clinicopathologic Variables of Hepatocellular Carcinoma**

| Clinicopathologic Variable | No. | Low | High | P^a |
|----------------------------|-----|-----|------|-----|
| Sex                        |     |     |      |     |
| Female                     | 11  |  4  |  7   | .863|
| Male                       | 49  | 17  | 32   |     |
| Age, y                     |     |     |      |     |
| ≤60                        | 48  | 17  | 31   | .813|
| >60                        | 12  |  4  |  8   |     |
| Tumor number               |     |     |      |     |
| Solitary                   | 29  | 16  | 13   | .012|
| Multiple                   | 31  |  5  | 26   |     |
| Liver cirrhosis            |     |     |      |     |
| Presence                   | 41  | 14  | 27   | .546|
| Absence                    | 19  |  7  | 12   |     |
| Vascular invasion          |     |     |      |     |
| Presence                   | 26  | 11  | 15   | .023|
| Absence                    | 34  | 10  | 24   |     |
| Edmondson-Steiner grade    |     |     |      |     |
| Low grade (I and II)       | 28  |  9  | 19   | .061|
| High grade (III and IV)    | 32  | 12  | 20   |     |
| Tumor size                 |     |     |      |     |
| ≤5 cm                      | 20  |  7  | 13   | .466|
| >5 cm                      | 40  | 14  | 26   |     |
| Capsular formation         |     |     |      |     |
| Presence                   | 28  | 11  | 17   | .037|
| Absence                    | 32  | 10  | 22   |     |
| Hepatitis B status         |     |     |      |     |
| Negative                   | 14  |  6  |  8   | .481|
| Positive                   | 46  | 15  | 31   |     |
| TNM stage (AJCC 7th)^b     |     |     |      |     |
| I                          | 25  | 13  | 12   | .020|
| II/III                     | 35  |  8  | 27   |     |

^a Bold values indicate statistical significance.

^b Tumor-node-metastasis stage is based on the American Joint Commission on Cancer classification, 7th edition.
stress fiber-like structures was disappeared in HCCLM3SIN1-RNAi and MHCC97-HSIN1-RNAi cells. These findings indicate that SIN1 is required for the reorganization of F-actin leading to the formation of the cell skeleton.

**SIN1 Facilitates Epithelial-Mesenchymal Transition in HCC**

SIN1 is identified as a key regulator in the Akt pathway by controlling Akt-Ser473 phosphorylation and activation, which is very important for epithelial-mesenchymal transition (EMT). Because AKT activation up-regulates snail expression and induces EMT, we hypothesize that SIN1 facilitates EMT in HCC by regulating Akt-Ser473 phosphorylation and activation. To test this hypothesis, we investigated the expression level of Akt and p-Akt in HCCLM3SIN1-RNAi, MHCC97-HSIN1-RNAi, HCCLM3NC, and MHCC97-HNC cells and the results showed that the depletion of SIN1 inhibited Akt phosphorylation and activation in HCC (Fig. 4A). The expression of Snail, Vimentin, MMP9 and N-cadherin was significantly lower in HCCLM3SIN1-RNAi cells than in HCCLM3NC cells. In contrast, the expression of E-cadherin was much higher in HCCLM3SIN1-RNAi cells than in HCCLM3NC cells. Similar to HCCLM3 cells, down-regulation of SIN1 in MHCC97-H cells reduced Snail, Vimentin, MMP9, and N-cadherin expression but increased E-cadherin expression (Fig. 4A,B). To confirm these results, immunohistochemistry was used to investigate the expression of SIN1, Snail, Vimentin, N-cadherin, MMP9, and E-cadherin in the same HCC tissue (Fig. 4C). The results showed that Snail, Vimentin, N-cadherin, and MMP9 had positive correlation with SIN1; however, E-cadherin had negative correlation with SIN1.

### TABLE 2. Univariable and Multivariable Analysis by a Cox Proportional Hazards Regression Model

| Variables                  | No. | Univariable Analysis HR (95% CI) | P<sup>a</sup> | Multivariable Analysis HR (95% CI) | P<sup>a</sup> |
|----------------------------|-----|---------------------------------|--------------|------------------------------------|--------------|
| Sex                        |     |                                  |              |                                    |              |
| Female                     | 11  | 1.187 (0.635-1.929)              | .423         | -                                  | NA           |
| Male                       | 49  | 1.387 (0.933-2.658)              | .236         | -                                  | NA           |
| Age, y                     |     |                                  |              |                                    |              |
| ≤60                        | 48  | 1.821 (1.103-2.698)              | .038         | 1.687 (0.935-2.662)                | .048         |
| >60                        | 12  | 0.808 (0.387-1.273)              | .258         |                                    | NA           |
| Tumor number               |     |                                  |              |                                    |              |
| Solitary                   | 29  | 2.678 (1.392-4.373)              | .003         | 2.536 (1.212-4.387)                | .038         |
| Multiple                   | 31  | 1.872 (1.107-2.936)              | .041         | 1.877 (1.061-3.010)                | NS           |
| Liver cirrhosis            |     |                                  |              |                                    |              |
| Absent                     | 19  | 1.168 (0.587-2.132)              | .331         | -                                  | NA           |
| Present                    | 41  | 1.394 (0.736-2.897)              | .047         | 1.538 (0.702-3.155)                | NS           |
| Edmondson-Steiner grade    |     |                                  |              |                                    |              |
| Low grade (I and II)       | 28  | 2.010 (1.010-4.173)              | .032         | 1.373 (1.112-4.208)                | .046         |
| High grade (III and IV)    | 32  | 1.132 (0.975-1.363)              | .218         |                                    | NA           |
| Tumor size                 |     |                                  |              |                                    |              |
| ≤5 cm                      | 20  | 1.394 (0.736-2.897)              | .047         | 1.538 (0.702-3.155)                | NS           |
| >5 cm                      | 40  | 2.401 (1.236-3.295)              | .039         | 2.236 (1.216-3.657)                | .037         |
| Hepatitis B status         |     |                                  |              |                                    |              |
| Negative                   | 14  | 1.187 (0.635-1.929)              | .423         | -                                  | NA           |
| Positive                   | 46  | 1.387 (0.933-2.658)              | .236         | -                                  | NA           |
| TNM stage (AJCC 7th)<sup>b</sup> | | | | | |
| I                          | 25  | 1.187 (0.635-1.929)              | .423         | -                                  | NA           |
| II/III                     | 35  | 1.387 (0.933-2.658)              | .236         | -                                  | NA           |

<sup>a</sup> Bold values indicate statistical significance.
<sup>b</sup> Tumor-node-metastasis stage is based on the American Joint Commission on Cancer classification, 7th edition.

Abbreviations: CI, confidence interval; HR, hazard ratio; NA, not adopted; NS, nonsignificant; SIN1, SAPK interacting protein 1.
Restoration of Snail Expression Can Imitate Function of SIN1 in Invasion and Migration of HCC Cells

To confirm whether SIN1 promotes invasion and metastasis of HCC by facilitating EMT, we transfected the Snail-plasmid into the HCCLM3SIN1-RNAi and MHCC97-HSIN1-RNAi cells to restore the Snail expression. The Transwell assay and wound healing assay were used to investigate the changes of invasion and migration ability after Snail was reintroduced into HCCLM3^{SIN1-RNAi} and MHCC97-H^{SIN1-RNAi} cells (Fig. 4D,E). The wound healing assay showed that cells closed faster after Snail was reintroduced into HCCLM3^{SIN1-RNAi} cells (92% versus 40%, P < .01), or MHCC97-H^{SIN1-RNAi} (86% versus 38%, P < .01). The Transwell assay also showed that the numbers of HCCLM3^{SIN1-RNAi} or MHCC97-H^{SIN1-RNAi} cells passed through the Matrigel was significantly increased after Snail was reintroduced (for

Figure 2. Immunohistochemistry of SAPK interacting protein 1 (SIN1) expression in hepatocellular carcinoma (HCC) tissues. (A–D) Immunohistochemistry was applied to examine the expression of SIN1 in HCC tissues. (A) SIN1-positive expression is shown. Magnification: ×400. (B) SIN1-negative expression is shown. Magnification: ×400. (C, D) In these representative images, SIN1 expression is high in HCC and is much more than that in adjacent nontumorous liver tissue (ANLT). Magnification: ×100 for (C), ×400 for (D). (E, F) Overall survival and disease-free survival was analyzed according to the expression of SIN1 in 60 cases of HCCs (using the Kaplan-Meier method). The results showed that patients with HCC who had high SIN1 expression have poorer overall survival than those with low SIN1 expression (P = .012). Analysis for disease-free survival shows the same results (P = .001).
HCCLM3SIN-RNAi cells: 148 ± 19 versus 37 ± 9, P < .01; for MHCC97-HSIN-RNAi cells: 131 ± 10 versus 35 ± 8, P < .01). These results showed that restored Snail expression can imitate the function of SIN1 in the invasion and migration of HCC cells, suggesting that SIN1 promotes invasion and metastasis of HCC by facilitating EMT.

DISCUSSION

SIN1 is essential for early embryonic development and is a key regulator of Akt phosphorylation and activation which play an important role in cancer.9-11 However, it is unclear whether SIN1 contributes to the development of human cancer. In the current study, we demonstrated for the first time that both SIN1 mRNA and protein increased significantly in HCC tissues compared with the corresponding ANLTs. Interestingly, SIN1 expression level in SLHCC was significantly lower than that in NHCC, but there was no significant differences between SLHCC and SHCC. We had found that SLHCC exhibits a low invasive and metastatic potential and a good outcome after resection.16,17 So these results indicate that SIN1 may play a critical role in invasion and metastasis of HCC. To confirm this finding, we examined SIN1 expression in HCC cells and found that SIN1 was overexpressed in HCC cell lines and correlated with the metastatic potential of HCC cells. Therefore, we further investigated whether SIN1 expression correlates with the clinicopathologic characteristics of HCC and found that SIN expression is associated with capsular formation, vascular invasion, and tumor numbers, indicating that SIN1 is required for the metastasis of HCC. We also demonstrated that SIN1 was associated with overall survival and disease-free survival in patients with HCC. Our results have demonstrated for the first time that SIN1 is increased in HCC and may play an important role in the metastasis of HCC.
Figure 4. SAPK interacting protein 1 (SIN1) facilitates epithelial-mesenchymal transition in hepatocellular carcinoma (HCC). (A) SIN1-shRNA was used to knockdown the expression of SIN1 in HCCLM3 and MHCC97-H cells. Western blot was employed to detect the expression level of p-AKT, AKT and Snail in each group. (B) Western blot was employed to detect the expression level of matrix metalloproteinase 9 (MMP9), Vimentin, E-cadherin, and N-cadherin in each group. (C) HCC tissues were immunolabeled for SIN1, Snail, N-cadherin, MMP9, Vimentin, and E-cadherin. Tissues in panels (a), (b), (c), (d), (e), and (f) showed positive staining (brown, cytoplasmic); tissues in panels (g), (h), (i), (j), and (k) showed negative staining. Snail, Vimentin, N-cadherin, and MMP9 showed positive correlation with SIN1; E-cadherin showed negative correlation with SIN1. (D) The Transwell assay was used to investigate the changes of invasion ability after Snail was reintroduced into HCCLM3SIN1-RNAi and MHCC97-HSIN1-RNAi cells. (E) The wound healing assay was used to investigate the changes of migration ability after Snail was reintroduced into HCCLM3SIN1-RNAi and MHCC97-HSIN1-RNAi cells.
To gain insight into the role for SIN1 in HCC metastasis, we employed shRNA to knockdown the SIN1 expression in HCCLM3 and MHCC97-H cells. Our results showed that depletion of SIN1 expression resulted in marked inhibition of cell invasion and migration in these 2 HCC cells. Furthermore, our findings indicate that suppression of SIN1 expression could significantly inhibit the reorganization of F-actin leading to the formation of stress fiber-like structures. These results confirmed that SIN1 could promote the invasion and metastasis of HCC.

It has been reported that SIN1 is a key TORC2 component and regulator of the Akt pathway that positively controls Akt phosphorylation and activation. It has been established that Akt phosphorylation and activation is very important for tumor metastasis.18-24 The Akt pathway could up-regulate snail expression and induces EMT,30-34 which plays a crucial role in the development of cancer metastasis.30-34 It is therefore of interest to investigate whether SIN1 promotes invasion and metastasis of HCC by regulating Akt pathway and facilitating EMT. Our results showed that down-regulation of SIN1 inhibited Akt phosphorylation and activation in HCC. Meanwhile, SIN1 depletion dramatically attenuated the expression of Snail and other mesenchymal markers such as Vimentin, MMP9, and N-cadherin. Conversely, SIN1 silencing increased the expression of E-cadherin, indicating that SIN1 might promote invasion and metastasis of HCC by facilitating EMT.

Because SIN1 promoted Akt phosphorylation, we studied the association of SIN1 expression with the expression of β-catenin signaling and mTOR signaling in HCCLM3 and MHCC97-H cell lines and HCC tissues. The results showed that SIN1 expression significantly affected mTOR signaling; however, there was no significant correlation between SIN1 and β-catenin signaling.

In conclusion, our study shows, for the first time, that SIN1 is overexpressed in HCC and its overexpression is significantly correlates with a poor prognosis of HCC. Furthermore, we have demonstrated that SIN1 plays an important role in HCC invasion and metastasis by facilitating EMT.

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CONFLICT OF INTEREST DISCLOSURE
The authors made no disclosure.

REFERENCES
1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin. 2011;61:69-90.
2. Forner A, Llovet JM, Bruix J. Hepatocellular carcinoma. Lancet. 2012;379:1245-1255.
3. Villanueva A, Hoshida Y, Battiston C, et al. Combining clinical, pathology, and gene expression data to predict recurrence of hepatocellular carcinoma. Gastroenterology. 2011;140:1501-1512.
4. Mayer J, Aurio J, Muscari F, et al. Wt3 prognosis of hypovascular hepatocellular carcinoma. J Hepatol. 2010;52:227.
5. Wang W, Wu F, Fang F, Tao YM, Yang LY. Inhibition of invasion and metastasis of hepatocellular carcinoma cells via targeting RhoC in vitro and in vivo. Clin Cancer Res. 2008;14:6804-6812.
6. Wu F, Yang LY, Li YF, Ou DP, Chen DP, Fan C. Novel role for epidermal growth factor-like domain 7 in metastasis of human hepatocellular carcinoma. Hepatology. 2009;50:1839-1850.
7. Fang F, Yang L, Tao Y, Qin W. FBL-1 promotes cell proliferation and enhances resistance to chemotherapy of hepatocellular carcinoma in vitro and in vivo. Cancer. 2012;118:134-146.
8. Fang F, Chang R, Yang L. Heat shock factor 1 promotes invasion and metastasis of hepatocellular carcinoma in vitro and in vivo. Cancer. 2012;118:1782-1794.
9. Jacinto E, Facchinetti V, Liu D, et al. SIN1/MIP1 maintains rictor-mTOR complex integrity and regulates Akt phosphorylation and substrate specificity. Cell. 2006;127:125-137.
10. Yang Q, Inoki K, Ikenoue T, Guan KL. Identification of Sin1 as an essential TORC2 component required for complex formation and kinase activity. Genes Dev. 2006;20:2820-2832.
11. Lazorchak AS, Liu D, Facchinetti V, et al. Sin1-mTORC2 suppresses rag and il7r gene expression through Akt2 in B cells. Mol Cell. 2010;39:433-443.
12. Fang F, Luo LB, Tao YM, Wu F, Yang LY. Decreased expression of inhibitor of growth 4 correlated with poor prognosis of hepatocellular carcinoma. Cancer Epidemiol Biomarkers Prev. 2009;18:409-416.
13. Li Y, Tang ZY, Ye SL, et al. Establishment of cell clones with different metastatic potential from the metastatic hepatocellular carcinoma cell line MHCC97. World J Gastroenterol. 2001;7:630-636.
14. Li Y, Tang Y, Ye L, et al. Establishment of a hepatocellular carcinoma cell line with unique metastatic characteristics through in vivo selection and screening for metastasis-related genes through cDNA microarray. J Cancer Res Clin Oncol. 2003;129:43-51.
15. Li Y, Tian B, Yang J, et al. Stepwise metastatic human hepatocellular carcinoma cell model system with multiple metastatic potentials established through consecutive in vivo selection and studies on metastatic characteristics. J Cancer Res Clin Oncol. 2004;130:460-468.
16. Wang W, Yang LY, Huang GW, et al. Genomic analysis reveals RhoC as a potential marker in hepatocellular carcinoma with poor prognosis. Br J Cancer. 2004;90:2349-2355.
17. Yang LY, Fang F, Ou DP, Wu W, Zeng ZJ, Wu F. Solitary large hepatocellular carcinoma: a specific subtype of hepatocellular carcinoma with good outcome after hepatic resection. Ann Surg. 2009;249:118-123.
18. Fang Y, Xue JL, Shen Q, Chen J, Tian L. MicroRNA-7 inhibits tumor growth and metastasis by targeting the phosphoinositide 3-kinase/Akt pathway in hepatocellular carcinoma. Hepatology. 2012;55:1852-1862.
19. Liu Y, Chen LF, Yuan YW, Li QS, Sun AM, Guan J. Activation of AKT is associated with metastasis of nasopharyngeal carcinoma. Tumor Biol. 2012;33:241-245.
20. Dey JH, Bianchi F, Voshol J, Benonfant D, Oakeley EJ, Hynes NE. Targeting fibroblast growth factor receptors blocks PI3K/AKT signaling, induces apoptosis, and impairs mammary tumor outgrowth and metastasis. Cancer Res. 2010;70:4151-4162.
21. Fu J, Chen Y, Cao J, et al. p28(GANK) overexpression accelerates hepatocellular carcinoma invasiveness and metastasis via phosphoinositide 3-kinase/AKT/hypoxia-inducible factor-1 alpha pathways. Hepatology. 2011;53:181-192.
22. Li B, Tsao SW, Li YY, et al. Id-1 promotes tumorigenicity and metastasis of human esophageal cancer cells through activation of PI3K/AKT signaling pathway. *Int J Cancer*. 2009;125:2576-2585.
23. Yang E, Boire A, Agarwal A, et al. Blockade of PAR1 signaling with cell-penetrating pepducins inhibits Akt survival pathways in breast cancer cells and suppresses tumor survival and metastasis. *Cancer Res*. 2009;69:6223-6231.
24. Sheng S, Qiao M, Pardee AB. Metastasis and AKT activation. *J Cell Physiol*. 2009;218:451-454.
25. Ogunwobi OO, Wang T, Zhang L, Liu C. Cyclooxygenase-2 and Akt mediate multiple growth-factor-induced epithelial-mesenchymal transition in human hepatocellular carcinoma. *J Gastroenterol Hepatol*. 2012;27:566-578.
26. Yoo YA, Kang MH, Lee HJ, et al. Sonic hedgehog pathway promotes metastasis and lymphangiogenesis via activation of Akt, EMT, and MMP-9 pathway in gastric cancer. *Cancer Res*. 2011;71:7061-7070.
27. Kang MH, Kang HN, Kim JL, Kim JS, Oh SC, Yoo YA. Inhibition of PI3 kinase/Akt pathway is required for BMP2-induced EMT and invasion. *Oncol Rep*. 2009;22:525-534.
28. Grille SJ, Bellacosa A, Upson J, et al. The protein kinase Akt induces epithelial mesenchymal transition and promotes enhanced motility and invasiveness of squamous cell carcinoma lines. *Cancer Res*. 2003;63:2172-2178.
29. Julien S, Puig I, Caretti E, et al. Activation of NF-kappaB by Akt upregulates Snail expression and induces epithelium mesenchyme transition. *Oncogene*. 2007;26:7445-7456.
30. Lee TK, Poon TP, Yuen AP, et al. Twist overexpression correlates with hepatocellular carcinoma metastasis through induction of epithelial-mesenchymal transition. *Clin Cancer Res*. 2006;12:5369-5376.
31. Thiery JP, Acloque H, Huang RY, Nieto MA. Epithelial-mesenchymal transitions in development and disease. *Cell*. 2009;139:871-890.
32. Tsuji T, Ibaragi S, Hu GF. Epithelial-mesenchymal transition and cell cooperativity in metastasis. *Cancer Res*. 2009;69:7135-7139.
33. Christiansen J, Rajasekaran AK. Reassessing epithelial to mesenchymal transition as a prerequisite for carcinoma invasion and metastasis. *Cancer Res*. 2006;66:8319-8326.
34. Singh A, Settleman J. EMT, cancer stem cells and drug resistance: an emerging axis of evil in the war on cancer. *Oncogene*. 2010;34:4741-4751.