HNRNPAB Induces Epithelial–Mesenchymal Transition and Promotes Metastasis of Hepatocellular Carcinoma by Transcriptionally Activating SNAIL

Zheng-Jun Zhou1,2, Zhi Dai1,2, Shao-Lai Zhou1,2, Zhi-Qiang Hu1,2, Qing Chen1,2, Yi-Ming Zhao1,2, Ying-Hong Shi1,2, Qiang Gao1,2, Wei-Zhong Wu1,2, Shuang-Jian Qiu1,2, Jian Zhou1,2,3, and Jia Fan1,2,3

Abstract

Expression of heterogeneous nuclear ribonucleoprotein AB (HNRNPAB) has been reported to be dysregulated in tumors, but its specific contributions to tumor formation and progression are not fully understood. Here, we demonstrate that HNRNPAB is overexpressed in highly metastatic cells and tumor tissues from patients with hepatocellular carcinoma (HCC) with recurrence. We found that HNRNPAB overexpression promoted epithelial–mesenchymal transition (EMT) in a manner associated with HCC metastasis in vitro and in vivo. RNA interference-mediated silencing of the EMT factor SNAIL attenuated HNRNPAB-enhanced cell invasion in vitro and lung metastasis in vivo. Mechanistically, HNRNPAB acted to transactivate SNAIL1 transcription, which in turn inhibited transcription of the pivotal SNAIL target gene E-cadherin. Overexpression of HNRNPAB in HCC samples correlated with higher SNAIL levels, shorter overall survival, and higher tumor recurrence. HNRNPAB overexpression, alone or in combination with SNAIL, was found to be a significant independent risk factor for recurrence and survival after curative resection. In conclusion, our findings define HNRNPAB as an activator of EMT and metastasis in HCC that predicts poor clinical outcomes.

Cancer Res; 74(10); 2750–62. © 2014 AACR.

Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related deaths worldwide (1, 2). Considering the high rate of tumor recurrence and metastasis after curative resection, understanding the mechanisms behind HCC metastasis and identifying new therapy targets are critical. Epithelial–mesenchymal transition (EMT), in which epithelial cancer cells lose their polarity and become motile mesenchymal cells, plays a pivotal role in dissemination of malignant hepatocytes during HCC progression (3–7). An increasing number of crucial EMT-related transcription factors, including SNAIL and SLUG, ZEB1/2, and TWIST, are involved in the EMT trigger during tumor progression (8–11). However, the molecular mechanisms that act upstream of these factors in various physiologic and pathologic contexts in HCC are not well characterized. Thus, uncovering the regulatory characteristics of these factors may enhance elucidation of the molecular mechanisms underlying HCC invasion and metastasis to develop novel therapeutic strategies.

Heterogeneous nuclear ribonucleoprotein AB (HNRNPAB) is a member of the hnRNP family of proteins that play important roles in normal biologic processes as well as cancer development (12–16). Together with its role in transactivation of the Ha-ras proto-oncogene, elevated HNRNPAB levels in solid tumor metastases strongly suggest a critical role in tumor progression (17, 18). Recently, several studies have reported that hnRNPs proteins may be master regulators of EMT. HNRNP2/B1, a member of the hnRNP subfamily, promotes EMT and lung cancer metastasis via regulation of E-cadherin expression (19). In addition, TGF-β–mediated phosphorylation of HNRNP1 induces EMT by transcript-selective translational induction of DAB2 and ILEI (20). Recent studies have also reported that HNRNPAB may play a critical role in tumor progression and malignant metastasis.

To date, no studies have reported the clinicopathologic significance of HNRNPAB in HCC. Here, we present the first
evidence that enforced expression of HNRNPAB induces EMT in HCC cells, which is accompanied by enhanced metastatic potential. We also reveal a crucial role for HNRNPAB in SNAIL-mediated E-cadherin suppression, HCC cell invasion, and lung metastasis. Furthermore, we show a positive correlation between HNRNPAB and SNAIL expression in HCC samples and their association with prognosis. Taken together, our data indicate that overexpression of HNRNPAB in HCC is a strong inducer of EMT and predicts a poor outcome in patients with HCC, and may serve as a potential therapeutic target.

Materials and Methods

Cell lines and animals

HepG2 and PLC/PRF/5 were purchased from the cell bank of Chinese Academy of Sciences (Shanghai, China). Cell lines were routinely checked for contamination by Mycoplasma using Hoescht staining, and were authenticated by DNA-Fingerprinting and isoenzyme analyses. These cell lines were obtained within 6 months before being used in this study. MHCC97H and HCCLM3 were established at our institute (25), and authenticated by short tandem repeat validation analysis during the study period. All cell lines were routinely maintained in high glucose Dulbecco’s Modified Eagle Medium supplemented with 10% FBS, at 37°C under 5% CO2 in incubator.

Male BALB/c nu/nu mice (4–6-weeks-old; Shanghai Institute of Material Medicine, Chinese Academy of Science) were raised in specific pathogen-free conditions. Animal care and experimental protocols were in accordance with guidelines established by the Shanghai Medical Experimental Animal Care Commission.

Patients and follow-up

Two independent cohorts of 417 patients with HCC were enrolled in this study. The 94 tumor tissues and paired adjacent nontumor cirrhotic liver tissues (cohort 1, snap-frozen tissues) were consecutively collected from patients undergoing curative resection from 2003 to 2004. The detailed information and follow-up procedures are described in the Supplementary Materials and Methods. Detailed clinicopathological features are listed in Supplementary Table S1. Ethical approval for the use of human subjects was obtained from the Research Ethics Committee of Zhongshan Hospital (Shanghai, China), and informed consent was obtained from each patient.

Lentivirus construction and cell transfection

The pGCSIL-shRNA-HNRNPAB/SNAIL lentiviral vectors were purchased from Shanghai Genechem Company Ltd. We constructed one FLAG-tagged HNRNPAB variant in the pcDNA3.1 vector, and this variant harbors a deletion of the RNA-binding domains (RBD) corresponding to the amino acids sequence of aa69-aa233. HNRNPAB cDNA vector and HNRNPAB variant were transfected into HepG2 cells, respectively. The pGCU lentiviral vector and pcDNA3.1 plasmid were used as controls. The transfected cells were selected under 800 μg/ml G418 (Sigma) for 3 to 5 weeks. Stably transfected clones were validated by qRT-PCR and Western blot analysis.

Cell proliferation, adhesion, migration, and Matrigel invasion assays

Cell proliferation was measured by BrdU-Assay Kit according to the manufacturer’ protocols (Roche). Cell adhesion assays were performed following the manufacturer’s instructions (Chemicon). Cell migration and Matrigel invasion assays were performed as described in Supplementary Materials and Methods.

In vivo assays for tumor growth and metastasis

The HCC cells were implanted subcutaneously into the flanks of nude mice, as described in the Supplementary Materials and Methods. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling staining was performed using kit (Roche), and was determined by counting the positive cells among the total number of cells in three to five randomly selected fields at \( \times 200 \). The proliferation index was determined by Ki-67 immunostaining and calculating the ratio of Ki-67-positive cells among total number of cells in three to five randomly selected fields at \( \times 200 \).

RNA isolation, qRT-PCR, immunofluorescence, and Western blot analyses

RNA isolation, qRT-PCR, immunofluorescence, and Western blot analyses were performed as previously described (26, 27). The HNRNPAB antibody (Catalog # SAB1100178; Gene ID, 3182) used in Western blot analysis test is purchased from Sigma-Aldrich Co. LLC. Other primers and antibodies used are listed in Supplementary Table S2 and Table S3, respectively.

Chromatin immunoprecipitation analysis

Chromatin immunoprecipitation (ChIP) assay was performed as described previously (21). Quantification of precipitated DNA by ChIP was performed using RT-PCR amplification. The primers used in the amplification are listed in Supplementary Table S2.

Plasmid construction

The SNAIL promoter construct was generated from human genomic DNA corresponding to the sequence from −2340/+146 of the 5’-flanking region of the SNAIL gene, as described in the Supplementary Materials and Methods. The 5’-flanking deletion constructs of the promoter (−1180/+146) and (−694/+146) were similarly generated with the (−2340/+146) SNAIL construct as a template. HNRNPAB-binding sites in the SNAIL promoters were mutated with a Site-directed Gene Mutagenesis Kit (Beyotime). Mutated SNAIL promoter and
pGL3-E-cadherin promoter constructs were cloned in the same manner. The constructs were confirmed by DNA sequencing. All of the primers are shown in Supplementary Table S2.

### Transient transfection and luciferase reporter assay

Transient transfection is included in the Supplementary Materials and Methods. The luciferase activity was detected with the Dual Luciferase Assay (Promega) according to the manufacturer’s instructions.

### Tissue microarray and immunohistochemistry

The construction of tissue microarray (TMA) and the immunohistochemistry protocols were described previously and the primary antibodies used are listed in Supplementary Table S3 (26). Images of representative fields were captured by a microscope (Leica) and analyzed using the Leica QWin Plus v3 software. Quantification of their expression levels were evaluated by Image-Pro Plus v6.0 software (Media Cybernetics). The mean densities were calculated as the ratio of integrated absorbance/total area. When HNRNPAB/ SNAIL was analyzed using immunohistochemistry, the median density was determined and used as a cutoff in subsequent analyses.

### Statistical analysis

Statistical analyses were performed using SPSS 16.0 for Windows (SPSS) as previously described (28). Quantitative data between groups were compared using the Student t test or Fisher exact test. Overall survival (OS) and cumulative recurrence rates were calculated by the Kaplan–Meier method and differences were analyzed by the log-rank test. Univariate and multivariate analyses were performed using the Cox proportional hazards regression model. P < 0.05 was considered statistically significant.

### Results

#### HNRNPAB is upregulated in human HCCs

To explore the role of HNRNPAB in HCC progression, we first evaluated HNRNPAB expression in various human HCC cell lines. We found that HNRNPAB expression was significantly increased in the highly metastatic cell lines (MHCC97H and HCCLM3) compared with the poorly metastatic HCC cell lines (HepG2 and PLC/PRF/5) at both the mRNA and protein levels (P < 0.01; Fig. 1A). Next, we evaluated expression of HNRNPAB in human HCC samples. qRT-PCR showed that nearly 67% of HCC tumors expressed high HNRNPAB mRNA levels compared with the matched peritumoral tissues (P < 0.05; Fig. 1B). Immunohistochemical analyses demonstrated predominant nuclear expression of HNRNPAB in HCC samples and support increased expression in the tumor compared with adjacent tissues (Fig. 1C). In addition, we observed higher HNRNPAB mRNA levels in patients with HCC recurrence compared with those without recurrence (mean HNRNPAB mRNA level 0.021 ± 0.26 in patients with recurrence vs. 0.011 ± 0.12 in those without recurrence, P < 0.05; Fig. 1D). Forty-eight cases of HCC used in Western blot analysis were randomly selected from 94 HCCs using computer-generated random numbers via SPSS software (Fig. 1E). The HNRNPAB protein levels were further found to be associated positively with recurrence by using Western blot and immunohistochemistry analyses (Fig. 1E and F). Thus, we used various approaches to demonstrate that HNRNPAB levels are frequently upregulated in human HCCs, particularly in patients with HCC with recurrence, indicating a potential role for HNRNPAB in HCC progression.

#### HNRNPAB promotes HCC cell invasion in vitro and lung metastasis in vivo

Stable upregulation of HNRNPAB expression in HepG2 cells and successful shRNA-mediated knockdown of HNRNPAB in HCCLM3 cells were confirmed using qRT-PCR and Western blot experiments (Fig. 2A and Supplementary Fig. S1A). In wound-healing migration assays, microscopic examination post-wounding revealed a significant delay in the wound closure rate of HCCLM3-hNRNPAB/shRNA-treated (HCCLM3-shRNA-hnRNPAB) cells compared with HCCLM3 cells (P < 0.05; Fig. 2B). In addition, HNRNPAB cDNA-transfected HepG2 (HepG2-hnRNPAB) cells had a significantly increased rate of wound closure compared with HepG2-mock cells (P < 0.05; Fig. 2B). In vitro invasive assays showed that the number of invasive cells in the HCCLM3-shRNA-hnRNPAB group was significantly decreased compared with the control (17.33 ± 7.19 vs.44.78 ± 11.51, P < 0.01; Fig. 2C and D), whereas the number of invasive cells in HepG2-hnRNPAB cells was significantly higher compared with control cells (24.22 ± 8.27 vs. 9.22 ± 5.74, P < 0.01; Fig. 2C and D). In addition, as shown in Supplementary Fig. S2E, HNRNPAB knockdown cells exhibited delayed cell proliferation in vitro when compared with control cells (P < 0.05).

After orthotropic transplantation of these cell lines into nude mice, all the groups successfully formed liver tumors. As shown in Supplementary Fig. S2G, the tumor size of HCCLM3-mock–derived xenografts was significantly larger than that of HCCLM3-shRNA-hnRNPAB–derived xenografts (3.31 ± 1.1 cm³ vs. 1.60 ± 0.48 cm³, P < 0.01). Similarly, the tumor size of HepG2-hnRNPAB–derived xenografts was 2.12 ± 0.7 cm³, which was markedly larger than that of HepG2-mock–derived tumors (1.40 ± 0.50 cm³, P < 0.05). A representative lung metastasis is shown in Fig. 2E. Pulmonary metastases occurred in 100% (7/7) of the HCCLM3-mock group. However, a lower rate was observed in the HCCLM3-shRNA-hnRNPAB group (29%, 2/7; Fig. 2F). In the HepG2-hnRNPAB mice, pulmonary metastases occurred in 57% (4/7), whereas no mice had metastases in the HepG2-mock group (0%, 0/7; Fig. 2F). In addition, we examined the cell proliferation index, apoptosis index, and microvessels number in solid tumors. As compared with the control groups, the HCCLM3-hnRNPAB-shRNA cell-derived tumors exhibited a decreased proliferation rate in vivo (number of Ki-67–positive cells, 12.00 ± 4.58 vs. 57.80 ± 5.54, P < 0.01; Supplementary Fig. S2A and S2B), whereas no significant differences in the apoptosis index or tumor microvessels (Supplementary Fig. S2A–S2D) were noted in tumors derived from these cells. Collectively, data from the in vitro and the in vivo assays show that HNRNPAB significantly contributes to tumor growth and metastasis of HCC.

**Zhou et al.**
HNRNPAB Induces EMT in HCC

We analyzed a panel of HCC cell lines with increased metastatic potential and found that elevated HNRNPAB levels in HCC cell lines correlated with the loss of E-cadherin and induction of vimentin (Supplementary Fig. S1C). Importantly, after transfection with HNRNPAB cDNA, HepG2 cells exhibited a spindle-like morphology (Fig. 3A). Upregulation of HNRNPAB in HepG2 cells led to significantly lower levels of an epithelial marker (E-cadherin) and high levels of a mesenchymal marker (vimentin), as shown by qRT-PCR, Western blot, and immunofluorescence analyses (Fig. 3B–E). HCCLM3 is a highly metastatic cell line that expresses low levels of E-cadherin and high levels of vimentin (Supplementary Fig. S1C), and is therefore thought to present a mesenchymal-like phenotype (4). As shown in Fig. 3A, distinct morphologic differences were observed between HCCLM3 cells and HCCLM3-shRNA-hnRNPAB cells. We observed reduced HNRNPAB levels in HCCLM3 cells, with a concomitant reduction of vimentin and upregulation of E-cadherin (Fig. 3B, C, and E). Moreover, changes in EMT marker expression following stable up- or downregulation of HNRNPAB were also evident in transplanted tumors of mice (Fig. 3F). Immunohistochemical.

Figure 1. HNRNPAB is upregulated in human HCCs. A, relative HNRNPAB levels in different HCC cell lines. Data represent the mean ± SD and are representative of three independent experiments. B, relative HNRNPAB levels among peritumoral and tumoral tissues using qRT-PCR. C and E, immunohistochemical staining analyses (C; scale bar, 100 μm) and Western blot analysis (E). D, the patients suffering from HCC recurrence exhibited higher mRNA HNRNPAB levels compared with patients without recurrence. Data, mean ± SD. E and F, protein levels of HNRNPAB in tumor samples of patients with HCC with or without recurrence by Western blot analysis and immunohistochemical staining analyses (scale bar, 100 μm).
analyses of HCC samples showed that patients with HCC with high HNRNPAB levels tended to have lower E-cadherin levels but higher vimentin levels (Fig. 6D). These data suggest that HNRNPAB is an inducer of EMT in HCC.

SNAIL mediates HNRNPAB-dependent regulation of E-cadherin expression

The EMT transition that is triggered during tumor progression is controlled by several key transcription factors, including SNAIL (SNAIL1), SLUG (SNAIL2), TWIST, and ZEB1/2, which act as transcriptional repressors of E-cadherin and induce EMT processes (8). qRT-PCR and immunoblot experiments showed that cellular levels of SNAIL were significantly increased after upregulation of HNRNPAB in HepG2 cells (P < 0.01; Fig. 3B and D). HNRNPAB knockdown in HCCLM3 cells resulted in downregulation of SNAIL expression (P < 0.01; Fig. 3B and D). However, the levels of other factors, including SLUG, TWIST, and ZEB1/2, were not significantly altered (Fig. 3B and D). Immunofluorescent analyses further confirmed higher SNAIL expression in HepG2-hnRNPAB cells compared with HepG2 cells (Fig. 3E). In contrast, E-cadherin expression was low in HepG2-hnRNPAB cells, however, was largely membrane localized in HepG2 cells (Fig. 3E). Moreover, changes in SNAIL expression following modification of HNRNPAB expression were evident in murine xenografts using immunohistochemical analyses (Fig. 3F). Thus, we speculated that SNAIL is critical for HNRNPAB-induced EMT processes in HCC.

To further validate the role of SNAIL in HNRNPAB-induced EMT, inhibition of SNAIL expression in HepG2-hnRNPAB cells increased E-cadherin expression compared with HepG2-hnRNPAB cells, despite high HNRNPAB levels.
Enforced SNAIL expression in HCCLM3-shRNA-hnRNPAB cells significantly decreased E-cadherin expression, despite repression of HNRNPAB expression (Fig. 4A). As shown in Supplementary Fig. S3A, we validated this proposed model in an additional two HCC cell lines: the low metastatic HCC cell line PLC/PRF/5 and a highly metastatic HCC cell line MHCC97H. Taken together, the above studies support a causal role for SNAIL in HNRNPAB-dependent changes in E-cadherin expression.

**SNAIL is critical for HNRNPAB-mediated HCC invasion and metastasis**

Previous studies have indicated that expression of SNAIL in HCC is associated with metastasis and prognosis (3, 29, 30). As shown in Fig. 4B, overexpression of SNAIL in HepG2 cells significantly increased cell invasion. After SNAIL knockdown in HCCLM3 cells, the number of invasive cell lines was approximately 50% of the control HCCLM3 cells (Fig. 4B and C). To investigate a possible role for SNAIL in HNRNPAB-induced cell invasion, shRNAs against SNAIL were transfected into HepG2-hnRNPAB cells. We found that this was sufficient to abolish the increased cell invasion produced by HNRNPAB overexpression (Fig. 4B and C). Conversely, upregulation of SNAIL in HCCLM3-shRNA-hnRNPAB cells partially reversed the inhibition of cell invasion produced by HNRNPAB knockdown (Fig. 4B and C). To determine the effect of SNAIL on HNRNPAB-mediated metastasis in vivo, we developed orthotopic HCC mouse models. In HepG2-hnRNPAB and HepG2-Snail mice, pulmonary metastases occurred in 57% (4/7) of mice, whereas only 14% (1/7) in the HepG2-hnRNPAB+shRNA-Snail group (Fig. 4D and E). The pulmonary metastasis rate of the HCCLM3-shRNA-hnRNPAB+Snail group was 86% (6/7), similar to HCCLM3 cells (Fig. 4D and E). The number of metastatic nodules of each grade in all transfected HCC cell lines were also measured and are shown in Fig. 4F. These results indicate that SNAIL plays a crucial role in HNRNPAB-mediated HCC invasion and metastasis.

**HNRNPAB regulates SNAIL mRNA transcription**

The above data suggest that HNRNPAB regulates SNAIL expression in HCC cells. Therefore, we assessed the possibility of hnRNPA-dependent regulation of SNAIL mRNA transcription. Using ChIP-PCR analyses in HCC cells, we found that HNRNPAB binds the SNAIL promoter (Fig. 5A). In promoter analyses, SNAIL and E-cadherin promoter luciferase constructs (pGL3-SNAIL and pGL3-E-cadherin) were transfected into HepG2-hnRNPAB cells, respectively. Luciferase reporter assays showed that HNRNPAB overexpression transactivated SNAIL promoter activity, thereby decreasing E-cadherin transcription (Fig. 5B). Furthermore, knockdown of SNAIL...
in HepG2-hnRNPAB cells partially relieved suppression of E-cadherin promoter-driven luciferase activity (Fig. 5B).

Multiple nucleic acid-binding tests of HNRNPAB show its ability to bind DNA at the canonical CarG box sequence (31, 32) and FTS-1 motif containing the core pentanucleotide sequence TTGAT (21). Sequence analyses of the SNAIL promoter regions showed that there are two putative HNRNPAB-binding sites (Fig. 5C, left). To define the cis-regulatory elements of the SNAIL promoter in response to HNRNPAB regulation, various constructs of the SNAIL 5'-flanking region were transfected into HepG2-hnRNPAB cells to determine the promoter transcriptional activities. Luciferase reporter assays showed that deletion from −2340 to −1180 had no significant effect on HNRNPAB-induced SNAIL promoter activity (Fig. 5C, right). Further deletion from −1180 to −694 significantly decreased HNRNPAB-induced SNAIL promoter activity (P < 0.01; Fig. 5C, right). In addition, we prepared mutant reporter constructs containing either deletions or substitutions in two putative HNRNPAB core-binding sites (−866 to −862 bp, designated site 1; or −1,980 to −1,976 bp, designated site 2), and then transfected these constructs into HepG2-hnRNPAB cells and parental HepG2 cells, respectively. As shown in Fig. 5C, deletion or substitution of site 1 significantly reduced the
luciferase activity of the SNAIL promoter (P < 0.01). Targeted deletion or substitution in site 2, however, had no significant effect on basal luciferase activity of the SNAIL promoter. Together with the data presented in Fig. 5, these findings suggest that HNRNPAB is a critical regulator of SNAIL gene expression, and specifically, /C0 to /C0 bp (designated site 1) is essential for HNRNPAB-induced SNAIL transactivation.

HNRNPAB expression positively correlates with SNAIL, whereas it inversely correlates with E-cadherin expression in HCC samples

On the basis of our novel findings, we assessed a potential clinical relationship among HNRNPAB/SNAIL/E-cadherin. Scatter plot analyses using qRT-PCR of HCC samples in cohort 1 revealed a significant positive correlation between HNRNPAB and SNAIL mRNA levels (Pearson correlation, n = 94, r = 0.634, P < 0.001; Fig. 6A, left), whereas a significant inverse expression pattern was noted for HNRNPAB versus E-cadherin (n = 94, r = −0.769, P < 0.001; Fig. 6A, right). Similar results were also observed at the protein level of HCC samples in cohort 2 (n = 323, P < 0.001; Fig. 6B). Using...
immunoblot analyses, we noted that in eight of 12 tumors, HNRNPAB and SNAIL correlate inversely with E-cadherin expression (Fig. 6C). Immunohistochemical analyses showed that patients with HCC with high HNRNPAB tended to have higher SNAIL levels but lower E-cadherin levels (Fig. 6D). In contrast, patients with HCC with low HNRNPAB may have lower SNAIL levels but higher E-cadherin levels (Fig. 6D). This specific expression pattern suggests a central role for HNRNPAB inducing EMT in HCC.

High HNRNPAB as well as HNRNPAB combined with SNAIL predict poor prognosis in patients with HCC

We investigated the expression of HNRNPAB and SNAIL using immunohistochemical staining in TMA composed of primary tumors and matched peritumoral tissues from 323 patients with HCC (cohort 2). Detailed clinicopathological features are listed in Supplementary Table S1. Immunohistochemical analyses revealed that 73% (236/323) of HCC tumors expressed high HNRNPAB protein levels compared with the corresponding peritumoral tissues (HNRNPAB protein mean density 0.059 ± 0.036 in HCC tumors vs. 0.032 ± 0.034 in peritumoral tissues, \( P < 0.01 \); Supplementary Fig. S1D). Among the above tumor samples, immunohistochemical analyses supported increased expression in patients with HCC recurrence compared with those without recurrence (HNRNPAB protein mean density 0.067 ± 0.041 in patients with recurrence vs. 0.050 ± 0.029 in those without recurrence, \( P < 0.01 \); Supplementary Fig. S1E). Taken together with the data presented in Fig. 1, we used an extensive collection of HCC tumors to show that HNRNPAB levels are frequently upregulated in human HCCs, particularly in patients with HCC with recurrence at both mRNA and protein levels.

Pearson \( \chi^2 \) tests indicated that HNRNPAB was significantly related to microvascular invasion (\( P = 0.009 \)), tumor encapsulation (\( P = 0.022 \)), \( \gamma \)-glutamyl transferase levels (\( P = 0.049 \)), and tumor-node-metastasis (TNM) stage (\( P = 0.006 \); Supplementary Table S4). SNAIL was significantly related to microvascular invasion (\( P = 0.003 \)), and TNM stage (\( P = 0.004 \); Supplementary Table S4). Furthermore, we used a median cutoff for HNRNPAB and SNAIL expression ("higher-than-median" indicates high expression and "lower-than-median" indicates low expression). We noted a trend toward better OS and lower recurrence rate for patients with HNRNPAB\textsuperscript{low} expression (Fig. 7A and B). We also observed significant OS and recurrence differences for patients according to SNAIL expression (Fig. 7C and D), which is consistent with previously reported results (29).

When HNRNPAB/SNAIL was analyzed using immunohistochemistry, the median density was determined and used as a cutoff in subsequent analyses. Hence, we divided all HCC samples into three groups: I, HNRNPAB\textsuperscript{low}/SNAIL\textsuperscript{low}; II, HNRNPAB\textsuperscript{low}/SNAIL\textsuperscript{high} and HNRNPAB\textsuperscript{high}/SNAIL\textsuperscript{low}; and III, HNRNPAB\textsuperscript{high}/SNAIL\textsuperscript{high}. Strikingly, patients with HCC expressing high levels of both HNRNPAB and SNAIL showed the worst prognoses (Fig. 7E and F). Conversely, patients with HCC who expressed low levels of both HNRNPAB and SNAIL had better prognoses (Fig. 7E and F). In addition, we found that the 1-, 3-, and 5-year OS rates of group I patients (81.7%, 72.2%, and 64.2%, respectively) were significantly higher than the OS rates for group II (75.3%, 53.8%, and...
49.4%, respectively) and group III patients (63.5%, 42.6%, and 29.5%, respectively; Fig. 7E). Moreover, the 1-, 3-, and 5-year cumulative recurrence rates in group I patients were 30.4%, 41.8%, and 44.7%, respectively, which were significantly lower than those for group II (41.4%, 56.0%, and 60.4%, respectively) and group III patients (53.2%, 71.8%, and 75.6%, respectively; Fig. 7F). A multivariate Cox proportional hazards model indicated that hnRNPAB and the coindex (hnRNPAB/Snail) were independent prognostic factors for OS ($P = 0.002$, HR = 1.685 and $P < 0.001$, HR = 2.257, respectively) and time to recurrence (TTR; $P = 0.011$, HR = 1.486 and $P < 0.001$, HR = 2.193, respectively; Supplementary Table S5). These data provide in vivo evidence that HNRNPAB and SNAIL may be useful biomarkers in HCC to indicate invasive tumor biology and poor prognosis.

**Discussion**

In this study, we used an extensive collection of HCC tumors to demonstrate that HNRNPAB is expressed at higher levels in HCC tissues than in peritumoral tissues and the samples from patients with recurrent HCC exhibited higher levels of HNRNPAB at both the mRNA and protein levels than patients without recurrence. Moreover, based on depletion and overexpression experiments in vitro and in vivo, we revealed a crucial role for HNRNPAB in regulating HCC invasiveness and metastasis.

Accumulating evidence from experimental and clinical studies suggests that EMT plays an important role in tumor invasion and metastasis (10, 34, 35). EMT is observed in subsets of carcinoma cells undergoing phenotypic conversion for invasion and metastasis and is characterized by the loss of epithelial cell junction proteins, such as E-cadherin, and the gain of mesenchymal markers, such as vimentin and N-cadherin (36). Here, we report that cells that express high levels of HNRNPAB exhibited amoeboid morphology and expressed high levels of vimentin and low levels of E-cadherin, suggesting that HNRNPAB may be a potent inducer of EMT, which may result in more invasive and metastatic HCC cells. Moreover, several recent reports support the hypothesis that the
mesenchymal–epithelial transition (MET) is essential for successful seeding and outgrowth of distant metastasis (10, 37). We examined expression of HNRNPAB and EMT markers expression in primary focus and metastatic lung nodules from xenograft tumor models using immunohistochemical tests. As compared with matched primary tumors, the tissue sections of lung metastases from HepG2-hnRNPAB and HCCLM3 groups, which expressed high levels of both HNRNPAB and SNAIL in primary tumors, showed lower expression of HNRNPAB, SNAIL, and vimentin as well as increased levels of E-cadherin (Supplementary Fig. S2H). These results suggest that MET might be essential for HNRNPAB-induced seeding and outgrowth of lung metastasis.

We have also presented data demonstrating a significant inverse relationship between expression of HNRNPAB and E-cadherin in HCC samples. In addition, manipulation of HNRNPAB in HCC cells inversely affects E-cadherin expression. Some previous reports linked loss of E-cadherin expression with HCC cell invasion and metastasis (4, 38, 39). Thus, we postulate that HNRNPAB-dependent regulation of E-cadherin expression is a key mechanism that contributes to the underlying loss of epithelial architecture and thereby helps initiate invasion. Transcriptional repression mediated by factors from the SNAIL (SNAIL1 and SLUG), ZEB (ZEB1 and ZEB2), and basic helix-loop-helix (such as TWIST) families is a common mechanism for the dynamic silencing of CDH1, the gene that encodes E-cadherin (8, 9, 11). Our study showed that HNRNPAB overexpression induces SNAIL expression, thereby repressing expression of E-cadherin. Interestingly, other transcriptional repressors of E-cadherin were not significantly affected by modulation of HNRNPAB expression in HCC cell lines. Moreover, we demonstrated a significant positive relationship between the expression of HNRNPAB and SNAIL in clinical HCC samples. SNAIL plays a prominent role in the negative regulation of E-cadherin expression and facilitates the EMT process (8, 11), which has been implicated in HCC metastasis and prognosis (3, 29, 30). In our study, we found that repression of SNAIL in HNRNPAB-overexpressing cells significantly suppressed HNRNPAB-enhanced cell invasion in vitro and lung metastasis in vivo. In addition, forced SNAIL expression in HNRNPAB knockdown cells partially reversed HNRNPAB-mediated inhibition of invasion and lung metastasis. Therefore, we conclude that HNRNPAB overexpression may promote HCC metastasis and EMT processes via SNAIL-mediated E-cadherin expression.

Important advances in our understanding of the post-transcriptional regulatory mechanisms of SNAIL have been reported (9, 40). Unfortunately, less is known about the transcriptional regulation of SNAIL. In this study, we demonstrated that HNRNPAB transactivates SNAIL expression by directly binding to its promoter, thereby inhibiting E-cadherin transcription. At this promoter, HNRNPAB may recognize DNA motifs with the canonical CArG box sequence and FTS-1 motifs containing the core pentanucleotide sequence TGTTA (21, 31). Our in silico search found that the promoter region of SNAIL contains two FTS-1 sites but does not contain a CArG motif. By using deletion or substitution mutants in a luciferase reporter assay, we showed that one of these FTS-1 elements is critical in HNRNPAB-dependent regulation of SNAIL mRNA transcription. In addition, HNRNPAB has been described as a transcriptional regulator that binds the FTS-1 element of the FSPI gene (21), and expression of this gene has been shown to be inversely correlated with E-cadherin expression. These properties are completely consistent with our identification of HNRNPAB as a transcriptional regulator of SNAIL, which mediates EMT and metastasis in HCC. Expression of SNAIL during EMT is controlled by alteration of several layers of regulation, including the transcriptional and translational machinery, noncoding RNAs, signaling pathways, and protein stability (40–45). These reports may explain the discordant expression levels of SNAIL and HNRNPAB in some HCC samples, as additional mechanisms may be involved in this process in these samples.

Another important study finding is the correlation between HNRNPAB expression and the prognosis for patients with HCC. Our survival analyses revealed that overexpression of HNRNPAB in HCC predicted shorter OS and higher recurrence rates. These findings strongly implicate HNRNPAB as a marker for tumor aggressiveness and a predictor for HCC survival. In summary, the roles of HNRNPAB in proliferation, invasion function, and pulmonary metastasis were responsible for the high recurrence rate and poor prognosis observed in HNRNPABhigh HCC patients. Our TMA results also confirmed that high SNAIL expression is a reliable indicator of poor prognosis for patients with HCC after resection. This finding is compatible with the findings of Yang and colleagues, which suggest that SNAIL is significantly related to microvascular invasion, and ultimately to metastasis (29). Importantly, patients with the HNRNPABhigh/SNAILhigh expression pattern were more likely to experience recurrence and suffered worse survival rates after curative resection.

In summary, our current findings demonstrate a novel role for HNRNPAB in the regulation of EMT via transcriptional activation of SNAIL. Overexpression of HNRNPAB in HCC is a strong indicator of aggressive tumors and poor clinical outcome. Uncovering novel functions and the underlying molecular mechanisms of HNRNPAB in HCC will shed new light on our understanding of EMT and tumor metastasis. In conclusion, our findings suggest that HNRNPAB may be a potential target for suppressing EMT under pathologic circumstances.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Z.-J. Zhou, Z. Dai, S.-L. Zhou, Z.-Q. Hu, Q. Gao
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Z.-J. Zhou, Z. Dai, S.-L. Zhou, Q. Chen, Y.-M. Zhao, Q. Gao
Writing, review, and/or revision of the manuscript: Z.-J. Zhou, Z. Dai, Q. Chen, W.-Z. Wu, S.-J. Qin, J. Zhou

2760 Cancer Res; 74(10) May 15, 2014 Cancer Research
hnRNPAB Induces EMT and Promotes HCC Metastasis

Grant Support
This work was supported by the National Basic Research Program of China (No.13ZR1452600), and the National Key Sci-Tech Project (2012ZX10002-003).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received September 3, 2013; revised February 28, 2014; accepted March 5, 2014; published OnlineFirst March 17, 2014.

References

www.aacnjournals.org

Cancer Res; 74(10) May 15, 2014 2761

Published OnlineFirst March 17, 2014; DOI: 10.1158/0008-5472.CAN-13-2509

Downloaded from cancerres.aacrjournals.org on November 14, 2017. © 2014 American Association for Cancer Research.
HNRNPAB Induces Epithelial–Mesenchymal Transition and Promotes Metastasis of Hepatocellular Carcinoma by Transcriptionally Activating SNAIL
