hnRNPAB induces epithelial-mesenchymal transition and promotes metastasis of hepatocellular carcinoma by transcriptionally activating Snail

Running head: hnRNPAB induces EMT and promotes HCC metastasis

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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 hepatocellular carcinoma (HCC) patients with recurrence. We found that hnRNPAB overexpression promoted epithelial-mesenchymal transition (EMT) in a manner associated with HCC metastasis in vitro and in vivo. RNAi-mediated silencing of the EMT factor Snail attenuated hnRNPAB-enhanced cell invasion in vitro and lung metastasis in vivo. Mechanistically, HnRNPAB acted to transactivate Snail 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.
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 is 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 physiological and pathological 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 in order 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 biological 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 hnRNP proteins may be master regulators of EMT. HnRNP2/B1, a member of the hnRNPA subfamily, promotes EMT and lung cancer metastasis via regulation of E-cadherin expression (19). Additionally, TGF-β-mediated phosphorylation of hnRNP1 induces EMT by transcript-selective translational induction of Dab2 and ILE1(20). Recent
studies have also reported that hnRNPAB may form a complex with KAP-1 to engage the transcription of FSP1 and play a key functional role in forming fibroblasts by EMT (21-22). HnRNPAB, in complex with actin, is involved in nuclear-cytoplasmic transport of mRNA(23), and is also associated with cytoskeletal changes in a mouse fetal cell line(24). These studies indicate that hnRNPAB might play a critical role in tumor metastasis and malignant progression.

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 HCC patients, 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 six months before being used in this study. MHCC97H and HCCLM3 were established at our institute (25), and
authenticated by STR validation analysis during the study period. All cell lines were routinely maintained in high glucose Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 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 HCC patients 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 in 2007 between January and June at our institute. The other cohort (cohort 2, n=323, paraffin-embedded tissues) were randomly collected from patients with HCC 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, 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., China. The designed target sequences are described in the Supplementary Materials and Methods. A negative control lentiviral vector containing
Non-silencing shRNA was constructed by a similar process. The shRNA sequence for the most efficient interference of hnRNPAB and Snail in HCC cells were confirmed by qRT-PCR and western blot.

The pGC-FU-hnRNPAB/Snail cDNA lentiviral vectors were purchased from Shanghai Genechem Company Ltd., China. We constructed one FLAG-tagged hnRNPAB variant in the pcDNA3.1 vector, and this variant harbors a deletion of the RBD domains corresponding to the amino acids sequence of aa69-aa233. HnRNPAB cDNA vector and hnRNPAB variant were transfected into HepG2 cells, respectively. The pGC-FU lentiviral vector and pcDNA3.1 plasmid were used as controls. The transfected cells were selected under 800μg/mL G418 (Sigma) for 3–5 weeks. Stably transfected clones were validated by qRT-PCR and western blot.

**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 was performed following the manufacturer’ 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 deoxyuridine triphosphate nick-end labeling staining was performed using kit (Roche), and was determined by counting the positive cells among total number of cells in 3-5 randomly selected fields at x200. The proliferation index was determined by
Ki67 immunostaining and calculating the ratio of Ki67-positive cells among total number of cells in 3-5 randomly selected fields at x200.

**RNA isolation, qRT-PCR and immunofluorescence, and western blot analyses**

RNA isolation, qRT-PCR, immunofluorescence and western blot were performed as previously described (26-27). The hnRNPAB antibody (Catalog # SAB1100179; Gene ID, 3182) used in western blot 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 (Chip) analysis**

Chip assay were performed as described previously(21). Quantification of precipitated DNA by ChiP was performed using real time 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 promotors were mutated with a Site-directed Gene Mutagenesis Kit (beyotime). And mutated Snail promoter and pGL3-E-cadherin promoter constructs was 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, USA) according to the manufacturer’s instructions.

TMA and immunohistochemistry

The construction of 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, USA), 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, USA). 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 cut-off in subsequent analyses.

Statistical analysis

Statistical analyses were performed using SPSS 16.0 for Windows (SPSS, Chicago, IL) as previously described(28). Quantitative data between groups were compared using the Student t test. Categorical data were analyzed by the chi-square test or Fisher exact test. 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

1. hnRNPAB is up-regulated 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 level (p<0.01; Fig. 1A). Next, we evaluated expression of hnRNPAB in human HCC samples. Quantitative realtime polymerase chain reaction (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 up-regulated in human HCCs, particularly in HCC patients with recurrence, indicating a potential role for hnRNPAB in HCC progression.

2. hnRNPAB promotes HCC cell invasion in vitro and lung metastasis in vivo.

Stable up-regulation 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 to 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 to 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 cm$^3$ vs. 1.60±0.48 cm$^3$, $p<0.01$). Similarly, the tumor size of HepG2-hnRNPAB-derived xenografts was 2.21±0.74 cm$^3$, which was markedly larger than that of HepG2-mock-derived tumors (1.40±0.50 cm$^3$, $p<0.05$). A representative lung metastasis is shown in Figure 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 to the control groups, the HCCLM3-hnRNPAB-shRNA cell-derived tumors exhibited a decreased proliferation rate in vivo (number of Ki67-positive cells, 12.00±4.58 vs. 57.80±5.54, p<0.01; Supplementary Fig. S2A and B), while no significant differences in the apoptosis index or tumor microvessels (Supplementary Fig. S2A-D) 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.

3. 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). Up-regulation 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, C, D, and 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 Figure 3A, distinct morphological 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 up-regulation of E-cadherin (Fig. 3B, C, and E). Moreover, changes in EMT marker expression following stable up- or down-regulation of hnRNPAB were also evident in transplanted tumors of mice (Fig. 3F). Immunohistochemical analyses of HCC samples showed that HCC patients 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.


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 up-regulation of hnRNPAB in HepG2 cells (p<0.01; Fig. 3B and D). HnRNPAB knockdown in HCCLM3 cells resulted in down-regulation 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 (Fig. 4A). 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.

5. **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 Figure 4B, overexpression of Snail in HepG2 cells significantly increased cell invasion. After Snail knockdown in HCCLM3 cells, the number of invasive cells was approximately 50% of the control HCCLM3 cells (Fig. 4B and C). To investigate a possible role for Snail in hnRNPAB-induced cell invasion, shRNA 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, up-regulation 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-hnRN-PAB+shRNA-Snail group (Fig. 4D and E). The pulmonary metastasis rate of the HCCLM3-shRNA-hnRN-PAB+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 Figure 4F. These results indicate that Snail plays a crucial role in hnRN-PAB-mediated HCC invasion and metastasis.

6. hnRN-PAB regulates Snail mRNA transcription.

The above data suggest that hnRN-PAB regulates Snail expression in HCC cells. Therefore, we assessed the possibility of hnRN-PAB-dependent regulation of Snail mRNA transcription. Using Chip-PCR analyses in HCC cells, we found that hnRN-PAB 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-hnRN-PAB cells, respectively. Luciferase reporter assays showed that hnRN-PAB overexpression transactivated Snail promoter activity, thereby decreasing E-cadherin transcription (Fig. 5B). Furthermore, knockdown of Snail in HepG2-hnRN-PAB cells partially relieved suppression of E-cadherin promoter-driven luciferase activity (Fig. 5B).

Multiple nucleic acid binding tests of hnRN-PAB 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 hnRN-PAB binding sites (Fig. 5C, left). To define the cis-regulatory elements of the Snail promoter in response to hnRN-PAB regulation, various constructs of the Snail 5’-flanking region were transfected into HepG2-hnRN-PAB 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 -1980 to -1976 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, -1180 to -694 bp (covering site 1) is essential for hnRNPAB-induced Snail transactivation.

HnRNPAB contains two RNA binding domain (RBDs) that mediate DNA/RNA binding, and followed by a C-terminal Gly-rich domain (33). To determine which region is required for its ability to transactivate Snail mRNA, we constructed one FLAG-tagged hnRNPAB variant in the pcDNA3.1 vector, and this variant harbors a deletion of the RBD domains. Following transfection of this construct into HepG2 cells, the expression of Snail and E-cadherin were not changed compared to that in control cells (Supplementary Fig. S3B). In addition, this variant had no effect on cell morphology (Supplementary Fig. S3C) or invasion ability of HCC cells in vitro (data not shown). When this variant was cotransfected
with pGL3-Snail into HepG2 cells, hnRNPAB showed no significant activity with the Snail promoter (Fig. 5B), suggesting the RBDs domain is necessary for hnRNPAB-mediated Snail transcription.

7. hnRNPAB expression positively correlates with Snail, whereas inversely correlates with E-cadherin in HCC samples.

Based on 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’s correlation, n=94, r=0.634, p<0.001; left, Fig. 6A), whereas a significant inverse expression pattern was noted for hnRNPAB vs. E-cadherin (n=94, r=-0.769, p<0.001; right, Fig. 6A). 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 HCC patients with high hnRNPAB tended to have higher Snail levels but lower E-cadherin levels (Fig. 6D). In contrast, HCC patients 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.

8. high hnRNPAB as well as hnRNPAB combined with Snail predict poor prognosis in HCC patients.

We investigated the expression of hnRNPAB and Snail using immunohistochemical staining in tissue microarrays (TMA) composed of primary tumors and matched
peritumoral tissues from 323 HCC patients (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 to 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 to 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 up-regulated in human HCCs, particularly in HCC patients with recurrence at both mRNA and protein level.

Pearson chi-square 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). And Snail was significantly related to microvascular invasion (\( p = 0.003 \)), and TNM stage (\( p = 0.004 \); Supplementary Table S4). Furthermore, we used a median cut-off for hnRNPAB and Snail expression ("higher-than-median" indicates high expression and "lower-than-median" indicates low expression). We noted a trend toward better overall survival (OS) and lower recurrence rate for patients with hnRNPABlow 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 cut-off in subsequent analyses. Hence, we divided all HCC samples into three groups: I, hnRNPAB\text{low}/Snail\text{low}; II, hnRNPAB\text{low}/Snail\text{high} and hnRNPAB\text{high}/Snail\text{low}; III, hnRNPAB\text{high}/Snail\text{high}. Strikingly, HCC patients expressing high levels of both hnRNPAB and Snail showed the worst prognoses (Fig. 7E and F). Conversely, HCC patients who expressed low levels of both hnRNPAB and Snail had better prognoses (Fig. 7E and F). And 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 co-index (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 \textit{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 results 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 TTGAT (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 FSP1 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, non-coding 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 HCC patients. 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 hnRNPAB\textsuperscript{high} HCC patients. Our TMA results also confirmed that high Snail expression is a reliable indicator of poor prognosis for HCC patients after resection. This finding is compatible with the findings of Yang \textit{et al.}, which suggest that Snail is significantly related to microvascular invasion and ultimately, to metastasis (29). Importantly, patients with the hnRNPAB\textsuperscript{high}/Snail\textsuperscript{high} 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.

References:

Figure Legends:

Figure 1. HnRNPAB is up-regulated 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) immunohistochemical staining analyses (scale bar: 100 μm) and (E) western blot analysis. (D) The patients suffering HCC recurrence exhibited higher mRNA hnRNPAB levels compared to patients without recurrence. Data represent the mean±SD. (E,F) Protein levels of hnRNPAB in tumor samples of HCC patients with or without recurrence by western blot analysis and immunohistochemical staining analyses. (scale bar: 100 μm).

Figure 2. HnRNPAB enhances metastatic potential of HCC cells.

(A) qRT-PCR and western blotting experiments showed up-regulation of hRNPAB in HepG2 cells and down-regulation of hRNPAB in HCCLM3 cells. Data represent the mean ±SD.(B) Wound-healing migration assays and the quantification of the percent of open area were shown. Data represent the mean±SD. Magnification, 200x. (C) Invasive behavior was tested using transwell matrigel invasion assays after overexpression or knockdown of hnRNPAB in HCC cells. Quantitation of tumor cell invasion is shown in (D). Magnification, 200x. Data shown are the mean±SD of at three independent experiments, each performed in triplicate. (E,F) Representative views of lung tissue sections from each group and the pulmonary metastasis rate are shown (scale bar: 100 μm). n=7.

Figure 3. HnRNPAB promotes EMT in HCC cells.

(A) Morphologic characteristics of HCC cell lines. (B) Western blot, (C) qRT-PCR, and (E) immunofluorescent staining show changes in EMT marker expression following stable up-
or down-regulation of hnRNPAB expression. Magnification, 200×. (B, D, and E) The effect of hnRNPAB on Snail, Slug, Twist, and Zeb1/2 expression were measured. Data represent the mean±SD. (F) Representative images from serial sections of tumor samples stained with hematoxylin and eosin (H&E), hnRNPAB, Snail, E-cadherin, and vimentin (scale bar: 100 μm). Data represent the mean±SD and are representative of three independent experiments.

**Figure 4. Snail is critical for hnRNPAB-mediated HCC invasion and metastasis.**

(A) HnRNPAB, Snail, and E-cadherin levels were evaluated in HCC cell lines transfected with the vectors as indicated. Data represent the mean±SD. (B and C) Snail knockdown significantly decreased hnRNPAB-enhanced cell invasion *in vitro*. Data shown are the mean±SD of at three independent experiments, each performed in triplicate. Magnification, 200×. (D) Snail knockdown suppressed hnRNPAB-mediated metastasis *in vivo*. n=7. Scale bar: 100 μm. (E) The incidence of lung metastases observed in the different groups of nude mice is shown. n=7. (F) The number of metastatic nodules of each grade in the different groups of nude mice was also measured. The metastases were classified into four grades on the basis of the number of tumor cells present at the maximal section for each metastatic lesion: grade I, ≤20 tumor cells; grade II, 20-50 tumor cells; grade III, 50-100 tumor cells; and grade IV, >100 tumor cells. Data represent the mean±SD.

**Figure 5. Snail is a direct transcriptional target of hnRNPAB.**

(A) Chip-PCR assays demonstrate the binding of hnRNPAB to the Snail promoter in HCC cells. We normalize each ChiP DNA fractions’ Ct value to the Input DNA fraction Ct value,
and calculated the %Input for each ChiP fraction. Data represent the mean±SD. (B)

HnRNPAB promoted Snail transcription but inhibited E-cadherin transcription. (C)

Deletion analysis and selective mutagenesis at position -866 to -862bp (designated site 1) or -1980 to -1976bp (designated site 2) site identified two hnRNPAB-responsive regions in the human Snail promoter. Serially truncated and mutated Snail promoter constructs along with the pRL-TK plasmid were transfected into HepG2-hnRNPAB cells and parental HepG2 cells, and then the relative luciferase activity was measured. The activity of each construct is presented relative to the activity of the Snail reporter gene in HepG2 cells. Data are expressed as the mean±SD. **, p<0.01. Schematic representations of the constructs are shown (left), and bar graphs show the relative level of luciferase activity in each sample (right). The boxes denote putative binding sites for hnRNPAB. The italic nucleotide sequences represent the core binding elements. The arrowes point to the corresponding position of the primers used in Chip-PCR assay.

Figure 6. HnRNPAB expression is positively correlated with Snail expression but inversely correlated with E-cadherin expression in human HCC tissues.

(A, B) The association between expression of hnRNPAB, Snail, and E-cadherin in HCC tissues. n=94 and n=323, respectively. (C) Western analyses of hnRNPAB, Snail, and E-cadherin expression in HCC tissues (n=12). Quantitation of the signal normalized against the internal control tubulin is shown. (D) Immunohistochemical analyses of hnRNPAB, Snail, E-cadherin and vimentin expression in HCC tissues (scale bar: 100 μm).

Figure 7. Kaplan-Meier analyses of overall survival (OS) and cumulative recurrence rate based on hnRNPAB and Snail expression.
(A-D) Compared with the hnRNPAB\textsuperscript{high} group, OS (A) and TTR (B) were significantly higher in the hnRNPAB\textsuperscript{low} group. Patients in the Snail\textsuperscript{low} group have higher OS (C) and TTR (D) when compared with the Snail\textsuperscript{high} group. (E, F) Prognostic values of hnRNPAB combined with Snail expression. I, hnRNPAB\textsuperscript{low}/Snail\textsuperscript{low}; II, hnRNPAB\textsuperscript{low}/Snail\textsuperscript{high} and hnRNPAB\textsuperscript{high}/Snail\textsuperscript{low}; III, hnRNPAB\textsuperscript{high}/Snail\textsuperscript{high}.
hnRNPAB induces epithelial-mesenchymal transition and promotes metastasis of hepatocellular carcinoma by transcriptionally activating Snail


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