Long noncoding RNA SchLAH suppresses metastasis of hepatocellular carcinoma through interacting with fused in sarcoma

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Emerging evidence has indicated that deregulation of long non-coding RNAs (lncRNAs) can contribute to the progression and metastasis of human cancer, including hepatocellular carcinoma (HCC). However, the roles of most lncRNAs in HCC remain largely unknown. Here we found a long noncoding RNA termed SchLAH (seven chromosome locus associated with HCC; also called BC035072) was generally downregulated in HCC. Low expression of SchLAH was significantly correlated with shorter overall survival of HCC patients. In vitro and in vivo assays indicated that overexpression of SchLAH inhibited the migration and lung metastasis of HCC cells. Knockdown of SchLAH by siRNA pool promoted the migration of HCC cells. RNA pull-down and RNA immunoprecipitation assays demonstrated SchLAH physically interacted with fused in sarcoma (FUS). PCR array analysis showed that RhoA and Rac1 were the downstream effector molecules of SchLAH during HCC metastasis. Knockdown of FUS rescued the mRNA levels of RhoA and Rac1 that were repressed by SchLAH. These results suggest that SchLAH may suppress the metastasis of HCC cells by interacting with FUS, which indicates potential of SchLAH for the prognosis and treatment of HCC.

Hepatocellular carcinoma (HCC) is currently the fifth most common tumor worldwide and the second leading cause of cancer-related death.1,2 Although significant progress in HCC treatment has been made in recent years, the 5-year survival rates are still low and approximately 422,100 HCC patients die each year in China.3,4 The poor prognosis and high recurrence rate is due to the high rate of metastases.5,6 However, the underlying molecular mechanisms that mediate the metastatic procedure remain unclear.7 Elucidation of the metastatic mechanisms may promote the development of effective diagnosis and treatment, and improve the overall prognosis of patients with HCC.

Long non-coding RNAs (lncRNAs) represent a subgroup of noncoding RNAs that are longer than 200 nucleotides. lncRNA-mediated biological regulation has been implicated in a wide variety of cellular processes, and in cancer lncRNAs are involved in multilevel regulation of gene expression,8,9 often by interacting with epigenetic complexes,10–12 proteins,13 miRNAs14,15 or mRNA.16,17 Recently, many studies have shown that lncRNAs are frequently deregulated in HCC and play important roles in cell proliferation,18–20 apoptosis,20–21 and metastasis.22,23 However, the clinical significance and roles of most deregulated lncRNAs in HCC remain largely unknown.

Fused in sarcoma/translocated in liposarcoma (FUS/TLS or FUS) is a multifunctional DNA/RNA-binding protein associated with cancer and neurodegeneration. Some articles revealed that FUS acted as a tumor suppressor and its
knockdown increased cell proliferation.\textsuperscript{(24,25)} Recently, FUS has been shown to interact with a number of lncRNAs in neuron diseases.\textsuperscript{(26)} Wang \textit{et al.}\textsuperscript{(27)} reported that FUS could be directed to the regulatory regions of target genes by lncRNA transcripts induced by DNA damage signals. However, relationship between FUS and lncRNAs in HCC has only been implicated in bioinformatic analysis\textsuperscript{(28)} and needs to further explore.

In this study, we first found a lncRNA termed \textit{SchLAH} that was downregulated in HCC tumor tissues, compared to the adjacent noncancerous tissues. Low expression of \textit{SchLAH} was correlated with poor prognosis of HCC patients. \textit{SchLAH} could suppress the migration of HCC cells \textit{in vitro} and lung metastasis \textit{in vivo}. Furthermore, we demonstrated that \textit{SchLAH} bound to FUS and inhibition of this binding between \textit{SchLAH} and FUS could contribute to promoting migration in HCC cells.

Materials and Methods

\textbf{Ethics statement}. The present study was approved by the research ethics committee of Zhongshan hospital, and the experiments were undertaken with the understanding and written consent of each subject.

\textbf{Patients and cell lines}. Frozen samples of HCC tissues and paired adjacent noncancerous liver tissues were randomly selected from patients undergoing hepatectomy at Zhongshan hospital (Shanghai, China) between 2004 and 2005. Ethical approval was obtained from the research ethics committee of Zhongshan hospital, and informed consent was obtained from each patient. All patients were followed up until October 2010. Overall survival (OS) was defined as the interval between the dates of surgery or the last follow-up.

\textbf{Cell culture}. The human HCC cell lines HepG2, Hep3B were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). HCC cell line SMMC7721 was purchased from Shanghai Institute of Cell Biology, Chinese Academy of Sciences. Huh7 was purchased from RIKEN BRC cell Bank, Tsukuba, Japan. All the cell lines were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U/mL penicillin, 100 mg/mL streptomycin), in a 5% CO\textsubscript{2} atmosphere at 37\textdegree C.

\textbf{Quantitative real-time PCR}. Real-time PCR analyses were performed according to the manufacturer’s instructions (Takara Biotechnology, Dalian, China). The primers used are listed in Table S1. The expression levels were normalized to \textit{\beta}-actin, and the relative expression levels were calculated using the 2\textsuperscript{-ΔΔCt} method.

\textbf{Western blotting}. Total cell lysates were prepared in 6× SDS loading buffer. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. Membranes were incubated with the specific primary antibodies and then with HRP-conjugated secondary antibodies. Protein bands were visualized using chemiluminescence detection. The following antibodies were used: FUS (1:500; Abcam, Cambridge, MA, USA), \textit{\beta}-actin (1:10 000; Sigma, St. Louis, MO, USA).

\textbf{Race}. 5’-RACE and 3’-RACE were performed using SMART RACE cDNA Amplification Kit (Clontech, Palo Alto, CA, USA) according to the manufacturer’s instructions. The gene-specific primers used for RACE analysis were presented in Table S2.

\textbf{SchLAH overexpression and RNA interference}. For overexpression, \textit{SchLAH} was cloned into pWPT vector. HepG2 and Hep3B cells were infected with virus containing the plasmid \textit{SchLAH}-pWPT. siRNA transfections were done with 50 nM siRNA pool (Ribobio, Guangzhou, China) and Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

\textbf{Subcellular fractionation location}. The separation of the nuclear and cytosolic fractions was performed using the PARIS Kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s instructions.

\textbf{Fluorescence in situ hybridization}. Cells were fixed in 4% formaldehyde then incubated in 5% acetic acid for 15 min followed by washes with phosphate-buffered saline (PBS). The fixed cells were further treated with pepsin (1% in 10 mM HCI) and subsequent dehydration through 70\%, 90\%, and 100\% ethanol. The air-dried slide was mounted with Prolong Gold Antifade Reagent with DAPI for detection. RNA FISH probes were designed and synthesized by Shanghai Biological Science and Technology Co., Ltd (Shanghai, China). Probe sequences are listed in Table S3.

\textbf{Transwell assays}. For the transwell migration assay, cells were trypsinized and resuspended in serum-free DMEM. 5 × 10\textsuperscript{4} cells (300 μL) were planted on the top chamber of each insert (BD Biosciences, Franklin Lakes, NJ, USA) with 8-μm-diameter pores on its membrane. To conduct migration assay of HCC cells, 800 μL DMEM supplemented with 10% FBS was injected into the lower chambers. After incubation at 37\textdegree C, cells remaining in the top chamber of the inserts were carefully removed. After fixation and staining in a dye solution containing 0.1% crystal violet, cells adhering to the lower side of the inserts were counted and imaged through an IX71 inverted microscope (Olympus, Tokyo, Japan).

\textbf{Cell proliferation assays}. Cell proliferation was assayed by Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan). HCC cells were plated in 96-well plates (1–2 × 10\textsuperscript{3} cells per well), and the numbers of cells per well were measured at the indicated time points.

\textbf{In vivo metastasis assays}. The tail vein metastasis model was established to validate the role of \textit{SchLAH} on HCC metastasis ability \textit{in vivo}. A total of 2 × 10\textsuperscript{4} HepG2 cells suspended in 200 μL serum-free DMEM were injected into the tail vein of nude mice. After 6 weeks, all of the mice were euthanized. The lung tissues were dissected and fixed with 4% formalin for at least 72 h. Lung tissues were analyzed by hematoxylin and eosin (HE) staining. SPF level BALB/c nude mice, male, age 6–7 week, weight 18–22 g, were provided by Shanghai Cancer Institute. The production license number is SCXX (Shanghai) 2007-0001 and the use certificate number is SYXX (Shanghai) 2007-0001. All mice were received human care and manipulated according to protocols approved by the Shanghai Medical Experimental Animal Care Commission.

\textbf{PCR array analysis for tumor migration}. The Cancer Motility RT\textsuperscript{2} Profiler PCR Arrays (Qiagen, Hilden, Germany), which is designed to represent 84 genes known to be involved in migration, was used to profile HepG2-SchLAH, Hep3B-SchLAH cells, and their relative control cells. PCR array was performed by Shanghai OE Biotech, Co, Ltd, Shanghai, China.

\textbf{RNA pull-down assay}. RNA pull-down and deletion mapping assays were performed as described previously.\textsuperscript{(10)} Briefly, biotinylated \textit{SchLAH} or antisense RNA was incubated with cell protein extractions (1 mg), which were then targeted with
strepavidin beads (Invitrogen) and washed. The associated proteins were resolved by gel electrophoresis. Specific bands were excised and identified by mass spectrometry. The length of synthetic biotinylated SchLAH is 2872 nt. Biotin RNA Labeling Mix (Roche diagnostics, Indianapolis, IN, USA) and DIG RNA Labeling Kit (SP6-T7) (Roche) were used to perform RNA pull-down experiment. The transcription buffer was from DIG RNA Labeling Kit (SP6-T7). Oligo Anealing buffer (Beyotime, Shanghai, China), RIPA lysis buffer (Beyotime), 5× RNA Oligo Anealing buffer (Beyotime) were used in this assay.

Chromatin immunoprecipitation and RIP. RIP assays were performed using a Millipore EZ-Magna RIP RNA-Binding Protein

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Fig. 1. Identification of SchLAH as a HCC-associated lncRNA. (a) SchLAH was downregulated in tumor tissues compared to nontumor liver tissues (GSE45436). (b–d) SchLAH was downregulated in tumor tissues compared to paired adjacent noncancerous liver tissues (n = 132). The expression level of SchLAH was analyzed by RT-PCR and normalized to β-actin. (e) Survival rates of 95 HCC patients who underwent liver surgery were compared between the SchLAH high-expression and SchLAH low-expression groups (log rank: P = 0.0124). The median expression level was used as the cutoff. (f) Schematic representation of SchLAH location on chromosome. (g) Fractionation of Huh7 and SMMC7721 cell lysates demonstrated nuclear expression of SchLAH. U1 RNA served as an internal control for nuclear gene expression. Values are mean ± standard deviation (n = 3). (h) Nuclear localization of SchLAH detected by RNA FISH in SMMC7721 and Huh7 cells. Scale bar, 10 μm.
Immunoprecipitation kit (Millipore, Bedford, MA, USA) according to the manufacturer’s instructions. RIP PCR was performed as RT-qPCR using total RNA as input controls. Antibodies used for RIP included rabbit polyclonal IgG (Millipore, PP64) and antibodies to FUS/TLS (Abcam, ab23439). The gene-specific primers used for detecting SchLAH are presented in Table S2.

**Statistical evaluation.** All statistical analyses were performed with SPSS 17.0 Software, Chicago, USA. Data were presented as mean ± standard deviation (SD) from at least three separate experiments. The significance of mean values between two groups was analyzed by Student’s t-test. Kaplan–Meier survival analysis was used to compare survival of HCC patients with high or low expression of SchLAH by log-rank test. A P-value < 0.05 was considered significant (*P < 0.05, **P < 0.01, and ***P < 0.001).

**Results**

SchLAH is downregulated in HCC tissues and associated with poor prognosis. By comparing HCC tissues to nontumor liver tissues using GEO database (GSE45436), we found a lncRNA
(NCBI: BC035072) was lowly expressed in HCC tissues ($P < 0.0001$) (Fig. 1a). To confirm expression of BC035072 in HCC, we examined 132 paired samples of tumor and nontumor liver tissues. Notably, BC035072 transcript was expressed at lower levels in over 90% of tumor tissues compared to the paired adjacent liver tissues of the same patients after normalizing to $\beta$-actin expression ($P < 0.0001$) (Fig. 1b–d). We next examined the relationship between expression levels of BC035072 and clinicopathological characteristics of 95 HCC patients (Table S4). Kaplan-Meier analysis showed significant correlations between the low expression level of BC035072 and poor overall survival ($P = 0.0124$) (Fig. 1e). Therefore, we focused on this uncharacterized lncRNA and named it SchLAH (seven chromosome locus associated with HCC) (Fig. 1f). SchLAH is composed of one exon with a full length of 2872 nt determined by RACE (rapid amplification of cDNA ends) assay (Fig. S1a,b). The sequence of SchLAH we obtained was identical with that in NCBI database.

Additionally, quantification of nuclear/cytoplasmic RNA in Huh7 and SMMC7721 cell extracts revealed that SchLAH transcripts were mainly enriched in the nucleus (Fig. 1g). We confirmed the localization of SchLAH in these cells using in situ hybridization assay (Fig. 1h). Open Reading Frame (ORF) confirmed the localization of SchLAH in situ hybridization assay (Fig. 1h). Open Reading Frame (ORF) finder analysis of the SchLAH sequence suggested that SchLAH had no coding potential. Consequently, SchLAH is a non-coding RNA in nuclei of HCC cells.

Overexpression of SchLAH inhibits migration of HCC cells in vitro and in vivo. The expression levels of SchLAH in HCC cell lines were detected by qRT-PCR (Fig. 2a). SchLAH level is relatively lower in HepG2 and Hep3B cells and higher in Huh7 and SMMC7721 cells. The hepatoma cell lines HepG2 and Hep3B were infected with the lentivirus containing the SchLAH expression vector (Fig. 2b). Notably, overexpression of SchLAH dramatically inhibited cell migration in vitro but did not affect cell proliferation and invasion (Fig. 2c,d, Fig. S2a,b). To further confirm the effects of SchLAH, we performed small antisense RNA-mediated knockdowns in SMMC7721 and Huh7 cells to observe the impact of SchLAH depletion. Silencing of SchLAH dramatically increased the ability of these cells to migration (Fig. 2e,f).

To test SchLAH in vivo, we established tail vein injected models and compared the rates of lung colonization as measured by hematoxylin and eosin (HE) staining. The number of micrometastatic lesions of each mouse was detected and counted under microscope. Compared to mice injected with HepG2 cells expressing a control vector, mice injected with HepG2 cells stably overexpressing SchLAH had fewer metastatic tumor clusters and smaller metastatic tumor size (Fig. 2g). These results indicate functional significance of SchLAH in HCC metastasis.

The interaction of SchLAH with FUS. Recently, several studies have found that many lncRNAs are involved in multiple regulation pathways through their interaction with proteins. SchLAH might affect cellular function in a similar manner. To test this hypothesis, we sought to identify proteins that are associated with SchLAH by an RNA pull-down experiment. We resolved the RNA-associated proteins on a SDS-PAGE gel, cut out the bands specific to SchLAH, and subjected them to mass spectrometry (Fig. 3a, Table S1). Among all of the proteins identified by mass spectrometry, only FUS was detected by western blotting from three independent RNA pull-down assays (Fig. 3b). To further validate the interaction between SchLAH and FUS, we performed RNA immunoprecipitation (RIP) with an antibody against FUS using cell extracts from SMMC7721 HCC cell lines. We observed an enrichment of SchLAH (but not other unrelated RNAs) with FUS antibody as compared to the nonspecific antibody (IgG control) (Fig. 3c). We further performed deletion-mapping experiments to determine whether FUS interacts with a specific region of SchLAH. We carried out RNA pull-down experiments with truncated versions of SchLAH followed by western blot detection of bound FUS (Fig. 3d). These analyses
identified a region between 800 and 1800 nt of SchLAH containing GGUG or GUGGU motif might be required for the interaction with FUS. As is reported, our results also indicate that FUS preferentially recognizes the UG-rich motif on RNA sequence.\(^{29}\)

**SchLAH** inhibits migration of HCC cells through RhoA and Rac1. To further investigate downstream molecular targets that were regulated by SchLAH in HCC migration, we analyzed migration-related genes for HepG2-SchLAH cells, Hep3B-SchLAH cells, and their relative control cells using a Cancer Motility RT² Profiler PCR Arrays (Fig. 4a). This analysis revealed a total of 11 downregulated migration-related genes in mRNA levels in HepG2-SchLAH cells and 12 in Hep3B-SchLAH cells, which had a more than 1.5-fold change, compared with their controlled cells, respectively (Fig. 4b). Among these genes, six genes were downregulated in both cell lines that overexpressing SchLAH. Subsequently, these six candidates were validated by qRT-PCR assay (Fig. 4c–f). When SchLAH was overexpressed, RhoA and Rac1 were notably downregulated, which was verified with the upregulation of RhoA and Rac1 when SchLAH was knocked down. These results indicate that RhoA and Rac1 may be the downstream targets of SchLAH during the metastasis of HCC.

**SchLAH functions through interaction with FUS.** FUS is a RNA-binding protein that is often associated with oncogenesis.\(^{30,31}\) FUS serves as a key molecule in transcriptional regulation and RNA processing including processes such as pre-messenger RNA (pre-mRNA) splicing and polyadenylation.\(^{32,33}\) Therefore, we sought to validate whether FUS is required for SchLAH to regulate the mRNA levels of RhoA and Rac1 indirectly. We performed siRNA against FUS in SchLAH overexpressed cells (Fig. 5a,b). Notably, depletion of FUS reversed the ability of SchLAH to suppress HCC cell migration (Fig. 5c,d). Quantitative RT-PCR confirmed that SchLAH-repressed target genes, such as RhoA and Rac1, were transcriptionally derepressed upon FUS depletion (Fig. 5e,f). These results suggest that FUS is required for SchLAH to suppress the migration of HCC cells and affects the expression of RhoA and Rac1 in an indirect way.

**Histone deacetylation is involved in the downregulation of SchLAH.** We next explored the reason for SchLAH downregulation in HCC. Recent study showed IncRNAs could be regulated by inhibitors of histone deacetylation in HCC cell lines.\(^{34}\) We determined that SchLAH was upregulated by the histone deacetylase inhibitor trichostatin A (TSA) in HepG2 (Fig. 6a) and Huh7 cells (Fig. 6b). To validate this result in vivo, we measured total histone H3 and H4 acetylation levels across the SchLAH promoter in HepG2 and Huh7 cells. Chromatin immunoprecipitation (ChIP) assay showed the enrichment of total histone H3 acetylation but not total H4 acetylation across the SchLAH promoter region (Fig. 6c). In addition, the histone H3 acetylation levels across SchLAH in HepG2 cells was lower than that in Huh7 cells which has

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*Fig. 4. SchLAH inhibits migratory potential of HCC cells through RhoA and Rac1. (a, b) Changes of gene expression were analyzed by a Cancer Motility RT² Profiler PCR array. The downregulated genes that fold change >1.5 in HepG2-SchLAH and Hep3B-SchLAH were intersected to generate an overlapping gene signature. (c–f) Overlapping genes in (b) were confirmed by qRT-PCR in HepG2, Hep3B, Huh7, and SMMC7721 cells. β-actin serves as a control. Values are mean ± standard deviation (n = 3). *P < 0.05, **P < 0.01.

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higher levels of this lncRNA. These results suggest that down-regulation of SchLAH may be caused by the H3 deacetylation.

Discussion

Metastasis and recurrence are the leading causes of poor survival of HCC patients. Although numerous genes involved in tumor metastasis were identified, metastatic mechanism of HCC remains poorly understood. Recently, increasing evidence has revealed functional roles of lncRNAs in cellular transformation and metastasis of HCC. In this study, we reported that SchLAH is frequently downregulated in 132 HCC tissues. With a cohort of 95 randomly selected HCC samples, we determined that low levels of SchLAH were associated with poor overall

![Fig. 5. SchLAH function through its binding to FUS.](image-url)
overexpression of SchLAH
However, it is not clear how through downregulating the mRNA level of RhoA and Rac1.

FUS was recruited by lncRNAs to the genomic locus encoding lncRNAs.(28) In this study, we identified a specific interaction between SchLAH and FUS. Wang et al. have shown that FUS preferentially interact with GUGGU RNA oligonucleotides. The latest cross linking-immunoprecipitation and high-throughput sequencing (CLIP-seq) data using mouse and human brain revealed the enrichment of GUGGU motif in FUS clusters.(36) Interestingly, SchLAH has several binding sites containing the FUS motif GGUG or GUGGU and our analyses indicate that SchLAH may bind to FUS through these motifs.

Fused in sarcoma is known to regulate transcription events through interaction with complexes such as transcription machinery.(37) The physical interaction between SchLAH and FUS is likely for SchLAH-mediated gene repression, as loss of FUS results in derepression of genes (such as RhoA and Rac1) that are repressed by SchLAH. Honda et al. revealed that “small GTPase-mediated signal transduction” was enriched in the list of the top 20 GO terms for genes with FUS-regulated expression in FUS-silenced neuron. It has been shown that RhoA and Rac1 were frequently upregulated in HCC tissue and is associated with poor prognosis. (39)

RhoA and Rac1 were frequently upregulated in HCC tissue and is associated with poor prognosis. (39) The expression of SchLAH was detected using qRT-PCR and normalized to β-actin. (c) ChIP analyses were conducted on the SchLAH promoter regions using anti-acetyl-histone H3 and anti-acetyl-histone H4. Enrichment was determined relative to input controls. Values are mean ± standard deviation (n = 3). *P < 0.05, **P < 0.01.

Survival. This result indicates that SchLAH may act as a tumor suppressor. We further observed that overexpression of SchLAH inhibited HCC cell migration in vitro and lung metastasis in vivo, while inhibition of SchLAH promoted HCC cell migration.

Many lncRNAs regulate gene transcription by interfacing with correspondent RNA binding proteins (RBPs) in a specific manner.(35) Computational analysis predicted that FUS is the most implicated RBPs in interaction with HCC related lncRNAs. In this study, we identified a specific interaction between SchLAH and FUS. Many lncRNAs regulate gene transcription by interfacing with correspondent RNA binding proteins (RBPs) in a specific manner. (35) Computational analysis predicted that FUS is the most implicated RBPs in interaction with HCC related lncRNAs. In this study, we identified a specific interaction between SchLAH and FUS. Wang et al. have shown that FUS preferentially interact with GUGGU RNA oligonucleotides. The latest cross linking-immunoprecipitation and high-throughput sequencing (CLIP-seq) data using mouse and human brain revealed the enrichment of GUGGU motif in FUS clusters. Interestingly, SchLAH has several binding sites containing the FUS motif GGUG or GUGGU and our analyses indicate that SchLAH may bind to FUS through these motifs.

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Lastly, our results indicated that SchLAH downregulation in HCC could be the result of histone H3 deacetylation. Epigenetic regulatory factors, such as histone acetylation and methylation or DNA methylation can manipulate the expression of lncRNA. (34,46,47) Yang showed that lncRNA-LET was repressed by hypoxia-induced histone deacetylase 3 (HDAC3). Our experiments also showed that the level of RhoA and Rac1 increased by use of TSA, while FUS slightly increased (Fig. S4). It is possible that RhoA and Rac1 were also regulated by histone deacetylase. In this case, it is hard to prove the overall hypothetical mechanism that overexpression of SchLAH could downregulate RhoA and Rac1 by TSA treatment. However, mechanisms of SchLAH downregulation in HCC needs to be further investigated.

Taken together, our results indicate that SchLAH, which is frequently downregulated in HCC, could promote HCC metastasis through interacting with FUS. These findings suggest that SchLAH may serve as an indicator for HCC prognosis and could be a potential therapeutic target.

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Disclosure Statement
The authors have no conflict of interest.

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Table S2. PCR primer pairs for qRT-PCR.

Table S3. Probes for RNA FISH.

Table S4. Clinical characteristics of 95 HCC patients according to SchLAH expression levels using β-actin as internal control.