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Reversing Effect of Ring Finger Protein 43 Inhibition on Malignant Phenotypes of human Hepatocellular Carcinoma

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Note: C. Xing and W. Zhou contributed equally to this study.
Abstract

It has been demonstrated that Ring finger protein 43 (RNF43) is overexpressed in colorectal cancer and mediates cancer cell proliferation; however, its role in hepatocellular carcinoma (HCC) remains unknown. In this study, we found that RNF43 was frequently overexpressed in HCC, and this overexpression was correlated with positive vascular invasion, poor tumor differentiation and advanced tumor stage. Functional studies showed that knockdown of RNF43 could induce apoptosis and inhibit proliferation, invasion, colony formation and xenograft growth of HCC cells. Microarray-based gene profiling demonstrated a total of 229 genes differentially expressed after RNF43 knockdown, many of which are involved in oncogenic processes such as cell proliferation, cell adhesion, cell motility, cell death, DNA repair and so on. These results suggest that RNF43 is involved in tumorigenesis and progression of HCC, and antagonism of RNF43 may be beneficial for HCC treatment.
Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide, with 748,300 new cases and 695,900 deaths annually (1). As existing therapies are insufficient for complete tumor eradication, the prognosis of HCC patients remains dismal. So it is crucial to unravel the molecular mechanisms of HCC and identify novel targets for therapeutic intervention that will improve the prognosis of HCC patients.

RING finger proteins are zinc finger variants characterized by the presence of the RING finger domain (2). They act mainly as ubiquitin-protein ligases that selectively mark proteins for degradation by the proteasome pathway (3). Previous studies have documented that RING finger proteins are involved in numerous biological processes, such as cell cycle, apoptosis, signal transduction and DNA repair (4-7). Ring finger protein 43 (RNF43) is a recently identified new member of Ring finger family that is highly expressed in human colorectal cancer (8). Forced overexpression of RNF43 has been shown to promote the growth of colon cancer cells, whereas knockdown of RNF43 retarded this growth (8). Besides its growth promoting effect, RNF43 also interacts with NEDL1, an upstream p53 regulator, thereby inhibiting the transcriptional and pro-apoptotic activity of p53 (9). Moreover, RNF43 could form structural complexes with PSF/p54nrb and HAP95 (10, 11), but the biological relevance of these physical interactions has not yet been proven.

Though RNF43 has been identified as an oncogene in colorectal cancer, its role in HCC remains unclear. In this study, we found that RNF43 was frequently
overexpressed in HCC, and its expression was correlated with poor clinical outcome. Furthermore, knockdown of RNF43 could inhibit growth, invasion and tumorigenicity of HCC cell lines. Using cDNA microarray analysis, a number of important signaling molecules were found to be involved in RNF43 regulatory network, and p53 was located at the center of this network.

Materials and methods

Patients

Ninety-eight pairs of primary HCC and adjacent noncancerous liver tissues were collected from patients who underwent hepatic resection between 2005 and 2010 in our hospital (First Affiliated Hospital, Zhejiang University School of Medicine, Zhejiang, China). These patients included 78 males and 20 females with a mean age of 51.4 ± 11.6 y (range: 24-76). Written informed consent was obtained from all patients, and the study was approved by the local ethics committee.

Cell culture

Ten Human HCC cell lines (HepG2, Hep3B, Huh-7, Bel-7402, SK-Hep-1, PLC/PRF/5, SMMC-7721, MHCC-97L, MHCC-97H and MHCC-LM3) and two immortalized liver cell lines (L-02 and Chang liver) were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco-Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (SAFC Biosciences, Lenexa, KS, USA), 100 units/mL penicillin and 100 mg/mL streptomycin (Sigma-Aldrich, St. Louis, MO, USA).
Small interfering RNA- or short hairpin RNA–based RNF43 knockdown

Three different small interfering RNAs (siRNAs) directed against human RNF43 and a scrambled siRNA were purchased from Shanghai GenePharma. The siRNA sequences are shown in Supplementary Table 1. To generate cells stably expressing RNF43 short hairpin RNA (shRNA), pGPU6/GFP/Neo-shRNF43 vector constructed by Shanghai GenePharma was transfected into HepG2 and SMMC-7721 cell lines with pGPU6/GFP/Neo-shNC vector as a negative control (Supplementary Table 1). Transfected cells were selected by 400 μg/mL G418 (Gibco-Invitrogen) for 3 weeks. Transfection of the siRNA or shRNA was performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions.

Quantitative Real-time PCR analysis

Total RNA was extracted from whole cells using Trizol reagent (Invitrogen) and reverse-transcribed with TaqMan Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Quantitative Real-time PCR (qRT-PCR) was carried out using SYBR Premix Ex Taq (TAKARA, Kusatsu, Japan) on an ABI 7500 Real Time PCR System (Applied Biosystems). All qRT-PCRs were performed in triplicate. The relative RNA expression was calculated using the delta-delta threshold cycle (ΔΔCT) method and normalized to GAPDH expression. Sequences of PCR primers are listed in Supplementary Table 1.

Immunohistochemistry

Immunohistochemistry (IHC) was performed as we have previously described (12). Primary antibodies and details are listed in Supplementary Table 2.
Western blotting analysis

Protein extraction and Western blotting analysis were performed as previously described (13). Primary antibodies and details are listed in Supplementary Table 2.

Cell viability assay

Cell growth was determined by Cell Counting Kit-8 (CCK-8) cell viability assay (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions as previously described (14).

Cell proliferation analysis

Cell proliferation was measured using the EdU assay kit (Ribobio, Guangzhou, China) according to the manufacturers' instructions as previously described (15).

Cell cycle analysis

Cell cycle analysis was carried out as previously described (14).

Cell apoptosis analysis

Cell apoptosis analysis was carried out as previously described (16).

Transwell invasion experiment

Forty-eight hours after siRNA transfection, 1 × 10^5 HepG2 cells or 5 × 10^5 SMMC-7721 cells in serum-free DMEM were seeded into the upper chambers of each well (24-well insert, 8-mm pore size, Millipore, Billerica, MA, USA) coated with Matrigel (BD Bioscience, San Jose, CA, USA). DMEM containing 10% FBS was placed in the lower chambers as a chemoattractant. After 16 hours of incubation, cells on the upper membrane surface were wiped off, and the cells that invaded across the Matrigel membrane were fixed with 100% methanol and stained with 0.2% crystal
violet. The number of invasive cells was then counted (five randomly chosen high-power fields for each membrane) under a microscope.

**Wound-healing experiment**

Wound-healing experiments were performed with Cytoselect 24-Well Wound-Healing assay (Cell Biolabs, San Diego, CA, USA) following the manufacturer’s instructions. Twenty-four hours after siRNA transfection, $1.5 \times 10^5$ cells were seeded in 24-well plates containing wound-healing inserts and incubated overnight. Inserts were removed generating a 0.9 mm open wound field in the monolayer of cells. Wound images were captured at 0 and 24 hours and the percentage of recovery was calculated as the ratio of the open area after and before wound closure.

**Cytoskeletal staining**

Cells were cultured on 4-well chamber slides (Millipore), fixed with 3.7% paraformaldehyde for 30 minutes, and permeabilized with 0.1% Triton X-100 for 5 minutes. Filamentous actin was stained with rhodamine-conjugated phalloidin (Sigma-Aldrich) in 1% bovine serum albumin in phosphate-buffered saline for 30 minutes at 37°C. The stained cells were visualized using an LSM510 Meta confocal microscope (Carl Zeiss, Jena, Germany).

**Extracellular matrix adhesion assay**

Cell-extracellular matrix (ECM) adhesion experiments were carried out using CytoSelect 48-well Cell Adhesion Assay ECM Array (Cell Biolabs) in accordance with the manufacturer's instructions. Seventy-two hours after siRNA transfection, $2 \times$
10^5 HepG2 cells or 8 × 10^5 SMMC-7721 cells in serum-free media were seeded into each well of the plates and were incubated at 37°C for 90 min. Media and nonadherent cells were aspirated, and the remaining adherent cells were incubated with stain solution for 10 minutes at room temperature. After washing the wells four times with deionized water, the cell stain was extracted and absorbance was recorded at 570 nm by a plate reader.

**Soft agar colony formation assay**

Colony formation in soft agar was performed using CytoSelect 96-Well Cell Transformation Assay (Cell Biolabs). Briefly, 2 × 10^3 cells were incubated 9 days in semisolid agar. Colony formation was then observed under a microscope and quantitated by the provided MTT solution following the manufacturer's instructions.

**Animal studies**

All animal studies were performed according to the Guide for the Care and Use of Laboratory Animals and with the approval of Institutional Animal Care and Use Committee. We purchased female BALB/c nude mice (4-5 weeks old) from Shanghai Experimental Animal Center of Chinese Academic of Sciences (Shanghai, China). Animals were kept under standard pathogen-free conditions and allowed to acclimate for 1 week prior to use. HepG2 cells (1 × 10^7/0.2 mL of phosphate-buffered saline) stably transfected with either control or RNF43 shRNA expression vectors were subcutaneously injected into the dorsal flank of each mouse (n = 5 mice/group). Tumor growth was monitored every 4 days using a caliper and the tumor volume was calculated with the following formula: Volume = π/6 × Length × Width^2. Four weeks
after injection, mice were sacrificed and xenografts were excised, fixed and paraffin embedded.

**TUNEL staining**

Paraffin-embedded slices of mice xenografts were deparaffinized, rehydrated, and transferase dUTP nick end labeling (TUNEL) assay was performed with an ApopTag kit (Millipore) following the manufacturer's instructions.

**Microarray experiments and data analysis**

HepG2 cells were transfected with RNF43 siRNA or negative control siRNA in triplicate. Forty-eight hours after transfection, total RNA was extracted using the RNeasy kit (Qiagen, Hilden, Germany) and integrity assessed with an Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA). The RNA was then labeled and hybridized to HG-U133 Plus 2.0 arrays (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's instructions. Raw microarray data were normalized using Affymetrix Expression Console Software Version 1.1 and Log 2-transformed. The transformed data of two groups were compared using Welch’s T-test. Differentially expressed genes were selected with a \( p \)-value of < 0.01 and a fold change of > 2. The Expression Analysis Systematic Explorer (EASE) software (http://david.abcc.ncifcrf.gov/ease/ease.jsp) was applied for Gene Ontology (GO) biological process enrichment analysis. Gene interaction network was constructed using the Genomatix Pathway System (GePS, http://www.genomatix.de/). The entire microarray data set is available at the GEO database (accession number GSE41326).
Statistical analysis

Microarray data analysis is described separately (see above). For the rest of the results, Pearson's chi-square test was applied to assess the correlation of RNF43 expression with different clinicopathological parameters. Independent Student's t test was used to analyze the differences between two groups. Statistical significance was accepted if $P < 0.05$. Statistical analysis was performed using SPSS 16.0 software (SPSS, Chicago, IL, USA). Data are presented as mean ± SD.

Results

Overexpression of RNF43 is frequent in HCC and is associated with poor clinical outcome

To determine the expression pattern of RNF43 in HCC, Oncomine database (17) was used to analyze two microarray data sets from the studies of Chen (18) and Mas (19). As seen in Figure 1A and B, the level of RNF43 mRNA was significantly higher in HCC than nontumor liver tissues ($P = 1.75 \times 10^{-13}$ and $P = 0.001$, respectively). To validate this observation, qRT-PCR was performed to evaluate the mRNA level of RNF43 in 12 HCC tissues, two immortalized liver cell lines (L-02 and Chang liver) and ten HCC cell lines (HepG2, Hep3B, Huh-7, Bel-7402, SK-Hep-1, PLC/PRF/5, SMMC-7721, MHCC-97L, MHCC-97H and MHCC-LM3). All samples, except SK-Hep-1, Bel-7402 and patient D58, showed at least four-fold overexpression of RNF43 compared with the mean value of L-02 and Chang liver cells (Figure 1C). IHC analysis of 98 pairs of primary HCC and adjacent normal tissues confirmed this
overexpression at protein level (Supplementary Figure S1), indicating the cancer specificity of RNF43 overexpression. Clinical association study demonstrated that this overexpression of RNF43 in HCC is associated with positive vascular invasion ($P < 0.001$), poor tumor differentiation ($P = 0.013$) and advanced tumor stage ($P = 0.027$, Table 1). Suggesting that RNF43 may contribute to the progression of HCC.

**Knockdown of RNF43 inhibits the growth of HCC cells**

To determine whether RNF43 knockdown could be used as effective therapy to treat HCC, further experimental studies were then performed. We employed three candidate siRNAs (RNF43-si1, -si2, -si3) to knockdown RNF43 in two HCC cell lines (HepG2 and SMMC-7721). Western blotting and qRT-PCR analysis exhibited that only RNF43-si1 could inhibit the expression of RNF43 effectively (Figure 2A and Supplementary Figure S2) and was therefore used for the following experiments. Transient knockdown of RNF43 resulted in a marked reduction of cell growth in both HepG2 and SMMC-7721 cells (up to 45% and 36%, respectively; Figure 2B), which was based on a decrease of proliferating cells (up to 44% in HepG2 and 56% in SMMC-7721; Figure 2C) and an increase of apoptotic cells (up to 44% in HepG2 and 56% in SMMC-7721; Supplementary Figure S3A and B). Cell cycle analysis revealed a major accumulation of G1 cells after RNF43 knockdown, along with a concomitant decrease of cell population in S phase (Figure 2D). Consistent with these findings, Western blotting analysis of several key proteins regulating the G1/S transition (including pRB, CDK2, CDK4, cyclin D1, cyclin D3 and p53) showed that under RNF43 knockdown conditions, the expression of the first five proteins was inhibited,
while that of p53 was up-regulated (Figure 2E).

**Knockdown of RNF43 inhibits invasion, migration and ECM adhesion of HCC cells**

As our study showed that RNF43 expression is positively correlated with vascular invasion, transwell assay was then performed to determine whether RNF43 knockdown would impact on the invasion of HCC cells. The result showed that siRNA-mediated knockdown of RNF43 significantly impaired the invasive capacity of HepG2 and SMMC-7721 cells (up to 81% and 64%, respectively; Figure 3A and Supplementary Figure S4A). It is worthy to note that the cells were only allowed to invade for 16 hours, and no significant difference in cell growth was observed at this time point (data not shown). Thus, the possibility that the cell growth could impact the invasion rate was excluded. Tumor cell migration is a prerequisite for invasion. We therefore investigated if this biological process were altered after RNF43 knockdown. Compared with the control group, cell migration ability was significantly decreased in HCC cells transfected with RNF43 siRNA (up to 51% in HepG2 and 52% in SMMC-7721; Figure 3B and Supplementary Figure S4B). Considering that actin cytoskeleton organization is essential for cell migration ability, the effect of RNF43 knockdown on actin stress fiber formation was then evaluated by phalloidin staining. As shown in Figure 3C, stress fiber formation was suppressed after RNF43 knockdown compared with control cells. The adhesion of cells to the extracellular matrix (ECM) is a critical requisite to generate cell shape and migration (20). Our results showed that RNF43 knockdown led to a moderate decrease in cell adhesion to
collagen I and collagen IV (Figure 3D and Supplementary Figure 4C), and this decreased adhesive property was associated with suppressed expression of trans-membrane protein integrin β4 (Supplementary Figure S4D). EMT is presently recognized as an important process for the development of cell invasion. However, our results indicated RNF43 knockdown was not sufficient to block EMT as characterized by suppression of E-cadherin expression (Supplementary Figure S4E) and change in morphology (not shown).

**Knockdown of RNF43 affects the expression of matrix metalloproteinases**

Matrix Metalloproteinase (MMP)-mediated ECM degradation plays a critical role in cell invasion. To assess the function of RNF43 knockdown in MMP induction, Western blotting was performed to detect the expression levels of MMP-1, -2, -3, -9, -11, -12, -13, -14, -17 and -21 72 hours after RNF43 knockdown. As shown in Supplementary Figure S5, siRNA-mediated RNF43 knockdown decreased MMP-3, -11, -12 and -14 protein levels while increasing that of MMP-1 and -9 in both HepG2 and SMMC-7721 cells. The protein levels of MMP -2, -13, -17 and -21 remained unchanged.

**Knockdown of RNF43 impairs tumorigenicity of HCC cells in vitro and in vivo**

Tumorigenicity is a hallmark of the malignant cancer cell. To determine the anti-tumorigenic effect of RNF43 knockdown, an RNF43 shRNA expression vector was established and stably transfected into HepG2 and SMMC-7721 cells, which effectively inhibited the expression of RNF43 (Figure 4A). The tumorigenic ability of RNF43 was then assessed by soft agar colony formation in vitro and by xenograft
growth in vivo. As shown in Figure 4B and C, HepG2 and SMMC-7721 cells with RNF43 knockdown displayed fewer and smaller colonies in comparison with blank vector transfected cells. Tumor xenograft studies demonstrated that knockdown of RNF43 could inhibit xenograft growth of HepG2 cells in nude mice, with a decrease of 77% in tumor volume after 4 weeks of implantation (Figure 4D and E). Histological analysis of xenograft sections revealed more apoptotic cells (12.74% ± 7.37% vs. 2.00% ± 1.19%, \(P=0.030\)), fewer mitotic cells (0.98% ± 0.46% vs. 1.92% ± 0.74%, \(P=0.042\)) and CD31-stained vessels (23.8 ± 12.4 vs. 48.4 ± 11.01 vessels per field, \(P=0.011\)) in RNF43 knockdown tumors compared with that of control groups (Supplementary Figure S6).

**Transcriptional changes in RNF43 knockdown cells**

To study the molecular mechanism of RNF43 knockdown, we profiled its gene expression pattern by microarray analysis, Affymetrix HG U133 2.0 plus was used to screen for global transcriptional changes in HepG2 cells 48 hours after RNF43 siRNA transfection. Overall, 229 genes were differentially expressed in RNF43 knockdown cells (including 95 up-regulated and 134 down-regulated genes, Supplementary Table 3). The differential expression of specific genes known to be highly implicated in cancer biology such as CDK2, CCNE2 and BAX were verified via qRT-PCR (Figure 5A). Gene ontology analysis showed that many differentially expressed genes are involved in biological processes relevant to cancer pathogenesis, such as cell proliferation, cell adhesion, cell motility, cell death, DNA repair and so on (Supplementary Figure S7). To obtain insights into the functional connections of
RNF43-regulated genes, these 229 genes were uploaded into the Genomatix GePS Software for network analysis. The network showed a dominance of p53, which functionally linked to 78 genes (Figure 5B). Some genes critically involved in cancer-relevant pathways were also highlighted as major nodes, such as JUN, EGFR and TGFB1, suggesting that these genes might act as important regulators in RNF43 regulating network.

Discussion

RNF43 is a recently identified member of the Ring finger family. Though it has been proven that RNF43 is highly expressed in colorectal carcinoma and acts as an oncogene in colon cancer cells (8, 9), its role in HCC has not been reported. Here, we found that RNF43 was frequently overexpressed in HCC tissues and cell lines, and its overexpression was correlated with positive vascular invasion, poor tumor differentiation and advanced tumor stage, suggesting that increased expression of RNF43 may contribute to the progression of HCC. To evaluate the therapeutic potential of RNF43 against liver cancer, we have then investigated whether knockdown of RNF43 could attenuate the malignant phenotypes (such as cell proliferation, apoptotic resistance and invasion) of HCC cell lines.

The proliferation and anti-apoptotic effects of RNF43 have been well established in colorectal cancer (8, 21). In keeping with previous reports, we found that knockdown of RNF43 by siRNA both inhibited proliferation and induced apoptosis in HCC cells. The underlying mechanism of RNF43 knockdown on cell proliferation is
not clear at present. Our results now demonstrate that knockdown of RNF43 can induce G1/S arrest. The expression alterations of pRB, CDK2, CDK4, cyclin D1, cyclin D3 and p53, which regulate the G1/S transition of cell cycle, matched well with the decreased proliferative status, suggesting that knockdown of RNF43 attenuates the activation of cyclin-cdk complexes, and therefore inhibit the proliferation of HCC cells. It is noteworthy that RNF43 has been reported to be implicated in p53-mediated apoptosis (9). In our study, the expression level of p53 was higher after RNF43 inhibition, suggesting that RNF43 knockdown induces HCC cell apoptosis, at least in part, via p53 dependent pathway.

Metastasis is a key event in tumor progression and is the most common cause of recurrence in HCC patients undergoing liver resection or transplantation (22, 23). Our clinical data has shown a positive association between RNF43 expression and metastatic potential of HCC. The functional study demonstrated that cell invasion was markedly inhibited after RNF43 knockdown, coincident with a reduction in both cell migration and ECM adhesion, reversely confirming the positive effect of RNF43 on HCC metastasis. Proteolysis of basement membrane is a crucial step in tumor invasion, which involves the activation of MMPs, a family of zinc-dependent endopeptidases capable of degrading all components of the ECM (24). Here, we found that the expression of MMP-3, -11, -12, -14 was down-regulated in RNF43 knockdown HCC cells, whereas that of MMP-1 and -9 was up-regulated. Overexpression of MMP-1 and -9 has been reported to be associated with high metastatic potential of HCC (25, 26). A more likely explanation of this conflicting
result is that RNF43 inhibition has a bifunctional role in the regulation of MMPs expression, and up-regulating the expression of MMP-3, -11, -12, -14 rather than down-regulating the expression of MMP-1 and 9 plays the dominant role, therefore suppresses the invasive ability of HCC cells. Furthermore, MMPs could also activate the release of bioactive fragments called matrikines during the degradation of ECM (27, 28), which interact with specific receptors such as integrin (29), leading to cellular adhesion and invasion (30-32). Integrins are a family of cell surface adhesion receptors. Here, we have found that integrin β4 was inhibited after RNF43 knockout. Integrin β4 is essential to carcinoma migration and invasion through its ability to regulate key downstream pathways including PI3K (33), Rac (34) and ERK1/2 (35), and overexpression of Integrin β4 is positively correlated with the invasive phenotype of several types of carcinomas (36). To our knowledge, this is the first report that RNF43 is also involved in tumor metastasis other than its proliferation and anti-apoptotic functions.

The *in vitro* experiments demonstrated an anti-oncogenic role of RNF43 knockdown in HCC and thus prompted us to address its *in vivo* functions in tumorigenesis. Our study showed that stable knockdown of RNF43 led to a significant inhibition of xenograft tumor growth in nude mice. Consistent with this finding, tumor sections from RNF43 knockdown xenografts exhibited lower levels of proliferative and anti-apoptotic activities. Remarkably, angiogenesis, a process essential for metastasis and growth of solid tumors, was also found to be inhibited in RNF43 knockdown xenografts. These results suggested that restricted proliferation
and angiogenesis activity combined with accelerated apoptotic process were attributed to the attenuated tumorigenicity of the RNF43 knockdown HCC cells \textit{in vivo}.

Finally, microarray analysis revealed that many genes involved in cancer-relevant processes (e.g. cell proliferation, cell adhesion and DNA repair) were differentially expressed after RNF43 knockdown. Functional network investigation of the microarray data highlighted p53 to be the central node in the RNF43 regulatory network, which exhibited the most interactions with other genes. It was recently reported that besides its anti-proliferative and apoptotic functions, p53 also negatively regulates cell motility and invasion (37), suggesting that p53 may involve in multiple aspects of RNF43’s oncogenic function. These findings provide a more comprehensive insight into the molecular mechanisms of RNF43 involved in HCC.

In conclusion, our results have shown that RNF43 is frequently up-regulated in hepatocellular carcinoma and is related to the aggressive phenotype of HCC. The functional data from both \textit{in vitro} and \textit{in vivo} investigations strongly suggest that knockdown of RNF43 could reverse the malignant phenotype of HCC. Furthermore, our study also generates a list of potential downstream target genes of RNF43, which provides valuable information for further investigation toward a comprehensive understanding of RNF43.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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References

### Tables

**Table 1.** Correlations between RNF43 expression and clinicopathological features.

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* Expression of RNF43 was graded according to the percentage of the stained cells as:
0 (<5%), 1+ (≥5% to <75%), 2+ (≥75%).

AFP, alpha-fetoprotein; HBsAg, hepatitis B surface antigen; Anti-HCV, hepatitis C infection.
Figure legends

Figure 1. RNF43 is overexpressed in HCC. Oncomine analysis of the microarray data from (A) Chen and (B) Mas. Whiskers, 10th and 90th percentile; box boundaries, 75th and 25th percentile; line within box, median. ***$P = 1.75 \times 10^{-13}$, **$P = 0.001$. (C) Relative RNF43 mRNA expression in ten HCC cell lines and 12 HCC tissues compared with the mean value of two normal liver cell lines (L-02 and Chang liver).

Figure 2. Knockdown of RNF43 inhibits the proliferation of HCC cells. (A) HepG2 and SMMC-7721 cells were transfected with RNF43 siRNAs (RNF43-si1, -si2, and -si3), negative control siRNAs (siNC), or Mock-transfected without RNA (Mock). Effects of siRNAs on RNF43 protein expression was detected via Western blotting. (B) HepG2 and SMMC-7721 cells were transfected with RNF43-si1 (siRNF43), and incubated for 72 hours before cell viability was assessed using CCK-8 assay kit (n=6, ***$P < 0.001$). (C) Cell proliferation was measured using EdU assay kit and quantified by counting the number of EdU-positive cells in five randomly chosen high-power fields (n=6, **$P < 0.01$, ***$P < 0.001$). (D) Flow cytometry showing that knockdown of RNF43 could arrest G1/S phase transition (n=3, *$P=0.023$, **$P < 0.01$). (E) Effects of RNF43 knockdown on protein expression of G1/S checkpoint regulators by Western blotting.

Figure 3. Knockdown of RNF43 inhibits invasion, migration and ECM adhesion of HCC Cells. (A) HCC cells were transfected with indicated siRNAs, cell invasion was measured by transwell invasion assay. Results were quantitated by counting invasive cells in five randomly chosen high-power fields for each replicate (n=3, *$P < 0.05$,
**P < 0.01). (B) Cell migration ability was assessed using wound-healing assay (n=3, *P < 0.05). (C) Actin stress fiber formation was detected by rhodamine-phalloidin staining. (D) Impact of RNF43 knockdown on adhesion of HepG2 cells to different ECM proteins (n=3, **P < 0.01, ***P < 0.001).

**Figure 4.** Knockdown of RNF43 impairs tumorigenicity of HCC cells. HCC cells were stably transfected with either RNF43 shRNA expression vector (shRNF43) or blank vector (shNC) as a negative control. (A) Suppression of RNF43 by shRNA was verified with Western blotting. Sh, RNF43 shRNA transfected; NC, negative control; P, parental cells. (B) Representative images of soft agar colony formation, and (C) the height of each bar shows the MTT absorbance representing the number of colonies in each group (n=6, **P < 0.01, ***P < 0.001). (D) RNF43 knockdown inhibited xenograft growth of HepG2 cells in nude mice (n=5, *P < 0.05, **P < 0.01, ***P < 0.001). (E) Images of xenografts in RNF43 knockdown group (upper panel) and control group (lower panel) at the end of the experiment.

**Figure 5.** Transcriptional changes in RNF43 Knockdown Cells. (A) Verification of differentially expressed genes after RNF43 knockdown by qRT-PCR. The vertical axis shows the log 2 ratio of RNF43 knockdown/control expression values. (B) Interaction network of differentially expressed genes between RNF43 knockdown and control groups. The red color indicates down-regulated genes, the green color indicates up-regulated genes, and the gray color indicates transcription factors, transcriptional downstream targets or genes that have frequent co-citations with input genes. Dashed line: interaction between co-cited genes; solid line: interaction between
genes with an expert curated connection. Genes that show no interaction with others are not displayed.
Figure 2

A

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B

Cell growth (OD value)

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C

Edu-positive cells (%)

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<tr>
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D

Cells in cell-cycle (%)

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</tr>
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E

<table>
<thead>
<tr>
<th>Protein</th>
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Figure 5

A

Fold change (log 2) of expression value

B

by default a gene product

kinase

phoshpatase

transporter

activation

inhibition

modulation