Companion Diagnostics & Cancer Biomarkers

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

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Abstract

It has been shown 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 HCCs, 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 HCCs. Microarray-based gene profiling showed 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 HCCs and that antagonism of RNF43 may be beneficial for HCC treatment.

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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 patients with HCCs remains dismal. So it is crucial to unravel the molecular mechanisms of HCCs and identify novel targets for therapeutic intervention that will improve the prognosis of patients with HCCs.

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 biologic 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 biologic relevance of these physical interactions has not yet been proven.

Although 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 HCCs, 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 HCCs and adjacent non-cancerous 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 years (range, 24–76 years). 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 2 immortalized liver cell lines (L-02 and Chang liver) were cultured in Dulbecco’s modified Eagle medium (DMEM; Gibco-Invitrogen) supplemented with 10% FBS (SAFC Biosciences), 100 units/mL penicillin, and 100 mg/mL streptomycin (Sigma-Aldrich).

siRNA- or short hairpin RNA-based RNF43 knockdown
Three different siRNAs directed against human RNF43 and a scrambled siRNA were purchased from Shanghai GenePharma. The siRNA sequences are shown in Supplementary Table S1. To generate cells stably expressing RNF43 short hairpin RNA (shRNA), pGP6/GFP/Neo-shRNF43 vector constructed by Shanghai GenePharma was transfected into HepG2 and SMMC-7721 cell lines with pGP6/GFP/Neo-shNC vector as a negative control (Supplementary Table S1). Transfected cells were selected with 400 μg/mL G418 (Gibco-Invitrogen) for 3 weeks. Transfection of the siRNA or short hairpin RNA (shRNA) was conducted with Lipotectamine 2000 (Invitrogen) 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). Quantitative real-time PCR (qRT-PCR) was carried out using SYBR Premix Ex Taq (Takara) on an ABI 7500 Real Time PCR System (Applied Biosystems). All qRT-PCRs were carried out in triplicate. The relative RNA expression was calculated using the delta delta threshold cycle (ΔΔCT) method and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. Sequences of PCR primers are listed in Supplementary Table S1.

Immunohistochemistry
Immunohistochemistry (IHC) was conducted as we have previously described (12). Primary antibodies and details are listed in Supplementary Table S2.

Western blotting analysis
Protein extraction and Western blotting were conducted as previously described (13). Primary antibodies and details are listed in Supplementary Table S2.

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

Cell proliferation analysis
Cell proliferation was measured using the EdU Assay Kit (Ribobio) according to the manufacturers’ instructions as previously described (15).

Cell-cycle analysis
Cell-cycle analysis was conducted as previously described (14).

Cell apoptosis analysis
Cell apoptosis analysis was conducted as previously described (16).

Transwell invasion experiment
Forty-eight hours after siRNA transfection, 1 × 10⁵ HepG2 cells or 5 × 10⁵ SMMC-7721 cells in serum-free DMEM were seeded into the upper chambers of each well (24-well insert, 8-mm pore size, Millipore) coated with Matrigel (BD Bioscience). 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 (5 randomly chosen high-power fields for each membrane) under a microscope.

Wound-healing experiment
Wound-healing experiments were carried out with Cytoselect 24-Well Wound-Healing Assay (Cell Biolabs) following the manufacturer’s instructions. Twenty-four hours after siRNA transfection, 1.5 × 10⁶ 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% parafomaldehyde 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 PBS for 30 minutes at 37°C. The stained cells were visualized using an LSM510 Meta confocal microscope (Carl Zeiss).

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 × 10⁵ HepG2 cells or 8 × 10⁵ SMMC-7721 cells in serum-free media were seeded into each well of the plates and were incubated at 37°C for 90 minutes. 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 4 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 conducted using CytoSelect 96-Well Cell Transformation Assay (Cell Biolabs). Briefly, 2 x 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 conducted 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 Academy of Sciences (Shanghai, China). Animals were kept under standard pathogen-free conditions and allowed to acclimate for 1 week before use. HepG2 cells (1 x 10^7/0.2 mL of PBS) stably transfected with either control or RNF43 siRNA 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 x length x width^2. Four weeks after injection, mice were sacrificed and xenografts were excised, fixed, and paraffin embedded.

Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling staining

Paraffin-embedded slices of mice xenografts were deparaffinized, rehydrated, and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay was conducted 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) and integrity assessed with an Agilent Bioanalyzer 2100 (Agilent Technologies). The RNA was then labeled and hybridized to HG-U133 Plus 2.0 arrays (Affymetrix) following the manufacturer’s instructions. Raw microarray data were normalized using Affymetrix Expression Console Software Version 1.1 and log2-transformed. The transformed data of 2 groups were compared using Welch t test. Differentially expressed genes were selected with P < 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 biologic process enrichment analysis. Gene interaction network was constructed using the Genomatix Pathway System (GePS, http://www.genomatix.de/). The entire microarray dataset 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 χ^2 test was applied to assess the correlation of RNF43 expression with different clinicopathologic parameters. Independent Student t test was used to analyze the differences between 2 groups. Statistical significance was accepted if P < 0.05. Statistical analysis was conducted using SPSS 16.0 software (SPSS). Data are presented as mean ± SD.

Results

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

To determine the expression pattern of RNF43 in HCCs, Oncomine database (17) was used to analyze 2 microarray datasets from the studies of Chen (18) and Mas (19). As seen in Fig. 1A and B, the level of RNF43 mRNA was significantly higher in HCCs than in nontumor liver tissues (P = 1.75 x 10^-13 and P = 0.001, respectively). To validate this observation, qRT-PCR was carried out to evaluate the mRNA level of RNF43 in 12 HCC tissues, 2 immortalized liver cell lines (L-02 and Chang liver) and 10 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 D8, showed at least 4-fold overexpression of RNF43 compared with the mean value of L-02 and Chang liver cells (Fig. 1C). IHC analysis of 98 pairs of primary HCC and adjacent normal tissues confirmed this.
overexpression at protein level (Supplementary Fig. S1), indicating the cancer specificity of RNF43 overexpression. Clinical association study showed that this overexpression of RNF43 in HCCs 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 HCCs.

**Knockdown of RNF43 inhibits the growth of HCCs**

To determine whether RNF43 knockdown could be used as effective therapy to treat HCC, further experimental studies were then conducted. We used 3 candidate siRNAs (RNF43-si1, -si2, -si3) to knockdown RNF43 in 2 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 (Fig. 2A and Supplementary Fig. 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 (~45% and 36%, respectively; Fig. 2B), which was based on a decrease of proliferating cells (~44% in HepG2 and 56% in SMMC-7721; Fig. 2C) and an increase of apoptotic cells (~44% in HepG2 and 56% in SMMC-7721; Supplementary Fig. S3A and S3B). Cell-cycle analysis revealed a major accumulation of G1 cells after RNF43 knockdown, along with a concomitant decrease of cell population in S-phase (Fig. 2D). Consistent with these findings, Western blotting 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 5 proteins was inhibited while that of p53 was upregulated (Fig. 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 conducted to determine whether RNF43 knockdown would impact on the invasion of HCCs. The result showed that siRNA-mediated knockdown of RNF43 significantly impaired the invasive capacity of HepG2 and SMMC-7721 cells (~81% and 64%, respectively; Fig. 3A and Supplementary Fig. 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 whether this biologic process was altered after RNF43 knockdown. Compared with the control group, cell migration ability was significantly decreased in HCCs transfected with RNF43 siRNA (~51% in HepG2 and 52% in SMMC-7721; Fig. 3B and Supplementary Fig. 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 Fig. 3C, stress fiber formation was suppressed after RNF43 knockdown compared with control cells. The

<table>
<thead>
<tr>
<th>Clinopathologic variable</th>
<th>RNF43 expression levels*</th>
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<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>In general</td>
<td></td>
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<tr>
<td>Normal adjacent tissue</td>
<td>59</td>
</tr>
<tr>
<td>Tumor tissue</td>
<td>18</td>
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<tr>
<td>Gender</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>12</td>
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<tr>
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<td>6</td>
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<tr>
<td>Age, y</td>
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<tr>
<td>$\leq$50</td>
<td>5</td>
</tr>
<tr>
<td>&gt;50</td>
<td>13</td>
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<tr>
<td>Liver cirrhosis</td>
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<tr>
<td>Absence</td>
<td>7</td>
</tr>
<tr>
<td>Presence</td>
<td>11</td>
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<tr>
<td>Maximal tumor size, cm</td>
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<tr>
<td>$\leq$5</td>
<td>5</td>
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<tr>
<td>&gt;5</td>
<td>13</td>
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<tr>
<td>Capsular formation</td>
<td></td>
</tr>
<tr>
<td>Absence</td>
<td>12</td>
</tr>
<tr>
<td>Presence</td>
<td>6</td>
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<tr>
<td>Tumor nodule number</td>
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</tr>
<tr>
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<td>Tumor stage (AJCC)</td>
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<td>I–II</td>
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<tr>
<td>III–IV</td>
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<td>Vascular invasion</td>
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<td>Presence</td>
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<tr>
<td>Tumor differentiation</td>
<td></td>
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</tr>
<tr>
<td>III–IV</td>
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<tr>
<td>Serum AFP, ng/mL</td>
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<tr>
<td>$\leq$400</td>
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<tr>
<td>&gt;400</td>
<td>7</td>
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<tr>
<td>HBsAg</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>2</td>
</tr>
<tr>
<td>Positive</td>
<td>16</td>
</tr>
<tr>
<td>Anti-HCV</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>18</td>
</tr>
<tr>
<td>Positive</td>
<td>0</td>
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</table>

*Expression of RNF43 was graded according to the percentage of the stained cells as 0 (<5%), 1+ (5%–<50%), 2+ (50%–<75%), 2+ (>75%).

Abbreviations: AFP, alpha-fetoprotein; HBsAg, hepatitis B surface antigen; anti-HCV, hepatitis C infection.
adhesion of cells to the 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 and collagen IV (Fig. 3D and Supplementary Fig. S4C), and this decreased adhesive property was associated with suppressed expression of transmembrane protein integrin β4 (Supplementary Fig. S4D). Epithelial–mesenchymal transition (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 Fig. 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 conducted 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 Fig. 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 (Fig. 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 Fig. 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 showed 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 (Fig. 4D and E). Histologic analysis of xenograft sections revealed more apoptotic cells (12.74% vs. 0.74%, P < 0.01) in RNF43 knockdown tumors compared with that of control groups (Supplementary Fig. 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 upregulated and 134 downregulated genes, Supplementary Table S3). The differential expression of specific genes known to be highly implicated in cancer biology such as CDK2, CCNE2, and BAX was verified via qRT-PCR (Fig. 5A). Gene ontology analysis showed that many differentially expressed genes are involved in biologic processes relevant to cancer pathogenesis, such as cell proliferation, cell adhesion, cell motility, cell death, DNA repair, and so on (Supplementary Fig. 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 (Fig. 5B). Some genes critically involved in cancer-relevant pathways were also highlighted as major nodes, such as JUN, EGRF, and TGFβ1, 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. Although 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 HCCs has not been reported. Here, we found that RNF43 was frequently overexpressed in HCCs 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 HCCs. 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 antiapoptotic 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 HCCs. The underlying mechanism of RNF43 knockdown on cell proliferation is not clear at present. Our results now show 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 HCCs. 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 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 patients with HCCs undergoing liver resection or transplantation (22, 23). Our clinical data has shown a positive association between RNF43 expression and metastatic potential of HCCs. The functional study showed 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 downregulated in RNF43 knockdown HCCs, whereas that of MMP-1 and -9 was upregulated. Overexpression of MMP-1 and -9 has been reported to be associated with high metastatic potential of HCCs (25, 26). A more likely explanation of this conflicting result is that RNF43 inhibition has a bifunctional role in the regulation of MMP expression, and upregulating the expression of MMP-3, -11, -12, -14 rather than downregulating the expression of MMP-1 and -9 plays the dominant role, therefore suppresses the invasive ability of HCCs. 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 knockdown. Integrin β4 is essential to carcinoma migration and invasion through its ability to regulate key downstream pathways including phosphoinositide 3-kinase (PI3K; ref. 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 antiapoptotic functions.
The *in vitro* experiments showed an anti-oncogenic role of RNF43 knockdown in HCCs 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 antiapoptotic 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 HCCs *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 antiproliferative and apoptotic functions, p53 also negatively regulates cell motility and invasion (37), suggesting that p53 may be involved in multiple aspects of the oncogenic function of RNF43. These findings provide a more comprehensive insight into the molecular mechanisms of RNF43 involved in HCCs.

In conclusion, our results have shown that RNF43 is frequently upregulated in hepatocellular carcinoma and is related to the aggressive phenotype of HCCs. The functional data from both *in vitro* and *in vivo* investigations strongly suggest that knockdown of RNF43 could reverse the malignant phenotype of HCCs. Furthermore, our study also generates a list of potential downstream effects.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: C. Xing, W. Zhou, B.-J. Wei, S. Zheng, L. Zhou


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Xing, W. Zhang, K. Chen, R. Su, J. Cheng, S. Zheng, L. Zhou

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Xing, Z. Yang, B.-J. Wei, K. Chen, R. Su, S. Zheng, L. Zhou

Writing, review, and/or revision of the manuscript: C. Xing, H. Xie, Z. Yang, S. Zheng, L. Zhou

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Xing, S.-M. Ding, H. Xie, S. Zheng, L. Zhou

Study supervision: C. Xing, H. Xie, S. Zheng, L. Zhou

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