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ORIGINAL ARTICLE – TRANSLATIONAL RESEARCH AND BIOMARKERS

Attenuated *RND1* Expression Confers Malignant Phenotype and Predicts Poor Prognosis in Hepatocellular Carcinoma

Hisateru Komatsu, MD[1,2], Tomohiro Iguchi, MD, PhD[1], Takaaki Masuda, MD, PhD[1], Hidenari Hirata, MD[1], Masami Ueda, MD[1,2], Shinya Kidogami, MD[1,2], Yushi Ogawa, MD[1], Kuniaki Sato, MD[1], Qingjiang Hu, MD[1], Sho Nambara, MD[1], Tomoko Saito, MD[1], Shotaro Sakimura, MD[1], Ryutaro Uchi, MD[1], Shuhei Ito, MD, PhD[1], Hidetoshi Eguchi, MD, PhD[1], Keishi Sugimachi, MD, PhD[1], Hidetoshi Eguchi, MD, PhD[2], Yuichiro Doki, MD, PhD[2], Masaki Mori, MD, PhD[2], and Koshi Mimori, MD, PhD[1]

[1]Department of Surgery, Kyushu University Beppu Hospital, Beppu, Japan; [2]Department of Gastroenterological Surgery, Graduate School of Medicine, Osaka University, Suita, Japan

ABSTRACT

Background. The *RND1* gene encodes a protein that belongs to the Rho GTPase family, which regulates various cellular functions. Depletion of *RND1* expression activates the oncogenic Ras signaling pathway. In this study, we aimed to clarify the clinical significance of *RND1* expression in predicting prognosis and to investigate its biological role in human hepatocellular carcinoma (HCC).

Methods. The association between *RND1* expression and clinical outcomes in patients with HCC was analyzed in three independent cohorts: 120 cases resected in our hospital; 370 cases in The Cancer Genome Atlas (TCGA); and 242 cases in GSE14520. Gene set enrichment analysis (GSEA) was also conducted. Finally, knockdown experiments were performed using small interfering RNA (siRNA) in vitro.

Results. In all cohorts, *RND1* expression was decreased as cancer progressed, and was affected by promoter methylation. In our HCC cases, the 5-year overall survival (OS) and recurrence-free survival of patients with low *RND1* expression was significantly poorer than those of patients with high *RND1* expression. TCGA and GSE14520 analyses provided similar results for OS. Multivariate analysis indicated that *RND1* expression was an independent prognostic factor for OS in all three cohorts. Additionally, GSEA showed an inverse correlation between *RND1* expression and the Ras signaling activity. In vitro, knockdown of *RND1* expression resulted in significant increases in proliferation, invasion, and chemoresistance to cisplatin in HCC cells.

Conclusions. Reduced *RND1* expression in HCC was associated with cancer progression, likely through regulation of the Ras signaling pathway, and may serve as a novel clinical biomarker for predicting prognosis in patients with HCC.

Hepatocellular carcinoma (HCC) is the most common histological subtype of liver cancer, accounting for approximately 70–90 % of all cases.[1] Although therapeutic strategies for HCC have been somewhat improved,[2] HCC is still one of the most incurable malignancies due to the limited availability of radical therapeutic approaches, and curative treatment is still restricted to surgical resection in most cases.[3,4] Thus, identification of novel biomarkers that can predict the prognoses of patients with HCC after surgery, and development of methods for the detection of molecules involved in tumor progression in HCC, are urgently needed.

The Rho family of GTPases is a family of small signaling G proteins involved in the regulation of common cellular functions, such as epithelial adhesion and polarity, cell migration, membrane trafficking, and cell cycle progression.[5,6] Rho GTPases are also known to play pivotal roles in cancer biology.[7] The Rho family GTPase 1 (*RND1*) gene, located at chromosome 12q13.12, encodes the RND1

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K. Mimori, MD, PhD
e-mail: kmimori@beppu.kyushu-u.ac.jp

protein, a member of the Rho GTPases.[8] A recent study in breast cancer demonstrated that *RND1* is a novel tumor-suppressor gene that blocks tumor initiation and progression via suppression of the Ras signaling pathway,[9] which is a highly activated oncogenic pathway involved in the progression of HCC.[10,11] However, the clinical and biological significance of *RND1* expression in HCC has not yet been elucidated.

In the current study, we investigated the clinical significance of *RND1* in three independent cohorts of HCC, and examined the biological role of *RND1* in tumor progression using HCC cells.

MATERIALS AND METHODS

All protocols in this study met the guidelines of the governmental agency and were approved by the Ethics Review Board of Kyushu University.

Patient and Sample Collection

Overall, 120 patients with HCC who underwent liver resection at Kyushu University Beppu Hospital and its affiliated hospitals (Oita Red Cross Hospital, Oita, Japan; Hiroshima Red Cross Hospital and Atomic-bomb Survivors Hospital, Hiroshima, Japan; and Iizuka Hospital, Fukuoka, Japan) between 2002 and 2005 were enrolled in this study. Tissues from resected tumors were immediately stored in RNAlater (Ambion, Austin, TX, USA), frozen in liquid nitrogen and kept at −80 °C until RNA extraction. Corresponding non-cancerous liver tissues were also collected (available in 58 of 120 cases). A 5-year follow-up was conducted after operation, and the average follow-up period for the 120 patients was 60.0 months (range 3.0–60.0). Patients were staged according to the 7th edition of the Union for International Cancer Control TNM classification system. Full written informed consent was obtained from all patients.

RNA Preparation, Reverse Transcription (RT) Polymerase Chain Reaction (PCR), and Quantitative Real-Time PCR

Total RNA from frozen tissue specimens and HCC cell lines was extracted using ISOGEN (Nippon Gene, Tokyo, Japan). The quality assessment of extracted RNA was performed by measuring absorbance, and we confirmed that all samples were of satisfactory quality. Complementary DNA (cDNA) was synthesized using reverse transcription polymerase chain reaction (RT-PCR) from 8 μg total RNA with M-MLV RT (Invitrogen, Carlsbad, CA, USA).

Quantitative real-time PCR (qRT-PCR) was performed using a LightCycler 480 Probe Master kit (Roche Applied Science, Basel, Switzerland). Gene expression was quantified using the following specific oligonucleotide primers: *RND1*: 5′-GCGAAGGATTGCTATCCAGA-3′ (sense) and 5′-GGTATCCCAGAGACTAAGCTCCA-3′ (antisense); *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*): 5′-AGCCACATCGCTCAGACAC-3′ (sense) and 5′-GCC CAATACGACCAAATCC-3′ (antisense). Messenger RNA (mRNA) amplification conditions consisted of initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 62 °C for 10 s, and elongation at 67 °C for 10 s. Melting curve analysis was performed to distinguish specific products from non-specific products and primer dimers. The relative expression levels of the gene were obtained by normalizing the amount of mRNA to that of *GAPDH* mRNA as an endogenous control in each sample.

Acquisition of Data on the Genetic and Epigenetic Profile of RND1 and Clinical Information from Public Hepatocellular Carcinoma (HCC) Datasets

We obtained paired RNA sequencing and survival data from 370 HCC cases from The Cancer Genome Atlas (TCGA), accessed via the Broad Institute's Firehose (http://gdac.broadinstitute.org/runs/stddata__2015_11_01/data/LIHC/20151101/). Of the 370 cases in TCGA, expression profiles of 50 paired non-cancerous liver samples were available. Data on single nucleotide polymorphism (SNP) arrays, methylation arrays, and whole-exome sequencing from the TCGA dataset were also obtained to investigate the genetic and epigenetic and regulation of *RND1* expression. DNA copy number was calculated by analyzing the SNP data, and its gains and losses were defined as log ratios of 0.15 or more and −0.15 or less, respectively. We also acquired mRNA expression profile data and clinical information on HCC cases from the National Cancer for Biotechnology Information Gene Expression Omnibus (accession code: GSE14520,[12,13] a total of 242 cases). Of the 242 cases in GSE14520, expression profiles of 228 paired liver samples were available.

Gene Set Enrichment Analysis

The statistical correlations between *RND1* expression profiles and predefined gene signatures in the public datasets were investigated with gene set enrichment analysis (GSEA)[14] using the TCGA and GSE14520 datasets. Details are provided in the electronic supplementary materials and methods.

Cell Lines

Established human HCC cell lines (Huh-7 and PLC/PRF/5) were used for this study. Details are provided in the electronic supplementary materials and methods.

Transfection with RND1-Specific Small Interfering RNA

Two types of *RND1*-specific small interfering RNAs (siRNAs) and a negative control siRNA were used for the transfection. Details are provided in the electronic supplementary materials and methods.

Western Blot Analysis

Proteins were detected using antibodies against RND1 (ab81143; Abcam, Cambridge, UK) and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Details are provided in the electronic supplementary materials and methods.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Assays

MTT assays were conducted using a Cell Proliferation Kit I (Roche Applied Science) to evaluate cell proliferation following the manufacturer's instructions. Details are provided in the electronic supplementary materials and methods.

Invasion Assays

Invasion assays were conducted using BD Biocoat Matrigel Invasion Chambers (pore size: 8 mm, 24-well; BD Biosciences, San Jose, CA, USA). Details are provided in the electronic supplementary materials and methods.

Growth Inhibition Assays with Cis-Diamminedichloro-Platinum (Cisplatin)

Growth inhibitory assays were performed using cisplatin (Wako Pure Chemical Industries, Osaka, Japan). Details are provided in the electronic supplementary materials and methods.

Statistical Analysis

For continuous variables, data were expressed as mean ± standard deviation, and statistical analysis was performed using Welch's *t*-tests. The degree of linearity was estimated by Spearman's rank correlation coefficient. Categorical variables were compared using Chi square tests or Fisher's exact tests. Overall survival (OS) and recurrence-free survival (RFS) were estimated using the Kaplan–Meier method, and the survival curves were compared using the log-rank test. Univariate and multivariate analyses were performed using the Cox proportional hazards model to identify independent variables predictive of OS. p values <0.05 were considered statistically significant. Data analysis of clinicopathological factors was performed using JMP 11 software (SAS Institute, Cary, NC, USA), and other analyses were performed using R version 3.1.1 (The R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

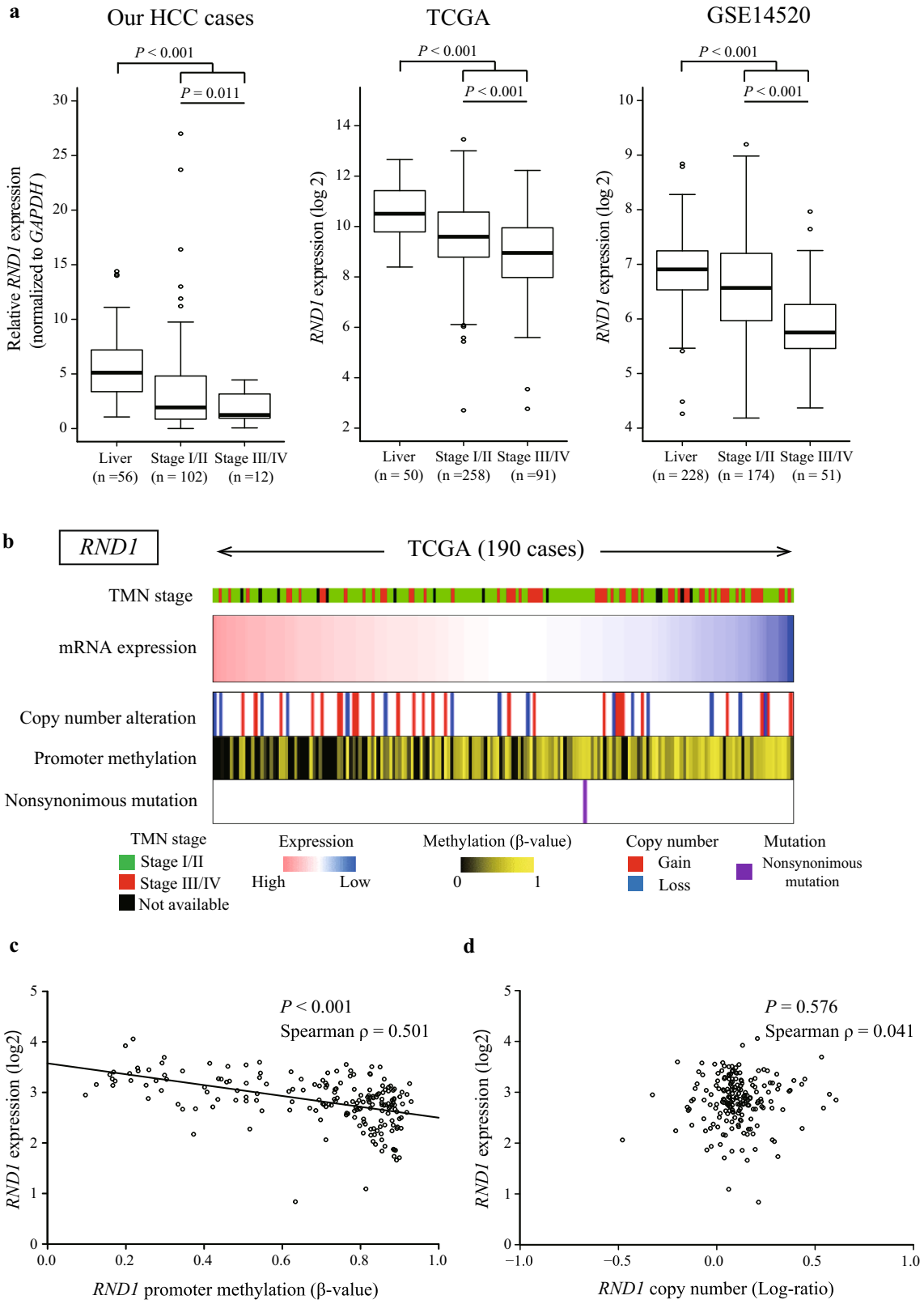
RND1 Expression was Reduced as HCC Progressed, and was Influenced by Gene Promoter Methylation

First, in order to clarify the clinical significance of *RND1* expression in HCC, we analyzed *RND1* expression in tumor tissues and non-cancerous liver tissues from three independent cohorts of HCC cases that contained information on TNM classification (our HCC cases: $n = 114$; TCGA: $n = 349$; and GSE14520: $n = 225$). In all three datasets, *RND1* expression in HCC was lower than that in liver tissue and was significantly downregulated in cases of advanced stages (TMN stage III/IV) compared with those of early stages (TMN stage I/II) (Fig. 1a).

Next, we further extracted gene promoter methylation, copy number, and somatic non-synonymous mutation data on *RND1* in TCGA (190 total cases) and conducted integrated analyses. We found that *RND1* expression was inversely correlated with the degree of *RND1* promoter methylation in tumors (Spearman's rank correlation coefficient -0.51; $p < 0.001$) (Fig. 1b, c), suggesting that *RND1* promoter methylation repressed the transcriptional activity of *RND1* in HCC. We also found that copy number alterations of *RND1* did not affect *RND1* expression (Fig. 1b, d). In addition, there was no association between *RND1* mutations and expression because only one non-synonymous mutation was observed in this analysis.

Low RND1 Expression Indicated Poor Prognosis and was Associated with Malignant Phenotype in HCC Cases

The three HCC cohorts were each divided into two groups according to *RND1* expression levels in HCC tissues using the minimum p value approach[15] for OS. In our HCC cases, OS and RFS in the low *RND1* expression group were significantly poorer than those in the high *RND1* expression group ($p = 0.001$ and 0.005, respectively) (Fig. 2a, b). For


a
Our HCC cases
TCGA
GSE14520
P < 0.001
P = 0.011
P < 0.001
P < 0.001
P < 0.001
P < 0.001
Relative RND1 expression (normalized to GAPDH)
RND1 expression (log 2)
RND1 expression (log 2)
Liver (n =56)
Stage I/II (n = 102)
Stage III/IV (n =12)
Liver (n = 50)
Stage I/II (n =258)
Stage III/IV (n =91)
Liver (n = 228)
Stage I/II (n = 174)
Stage III/IV (n = 51)
b
RND1
TCGA (190 cases)
TMN stage
mRNA expression
Copy number alteration
Promoter methylation
Nonsynonimous mutation
TMN stage
Stage I/II
Stage III/IV
Not available
Expression
High
Low
Methylation (β-value)
0
1
Copy number
Gain
Loss
Mutation
Nonsynonimous mutation
c
P < 0.001
Spearman ρ = 0.501
RND1 expression (log2)
RND1 promoter methylation (β-value)
d
P = 0.576
Spearman ρ = 0.041
RND1 expression (log2)
RND1 copy number (Log-ratio)

◀**FIG. 1** Downregulation of *RND1* expression via gene promoter methylation was associated with tumor progression in HCC cases. **a** Box plots of *RND1* expression in non-cancerous liver and tumor tissues grouped into early stages (I/II) and advanced stages (III/IV) in three independent cohorts of HCC cases. Information on tumor stage was available for 114 of 120 patients in our HCC cases (*left*), 349 of 370 cases in the TCGA dataset (*middle*), and 225 of 242 cases in the GSE14520 dataset (*right*). **b** An integrative view of mRNA expression, promoter methylation, copy number, and non-synonymous somatic mutation profiles of *RND1* across 190 HCC cases in TCGA. The samples are sorted according to *RND1* expression levels. **c** A correlation plot between *RND1* promoter methylation and expression in the TCGA dataset. **d** A correlation plot between *RND1* copy number and expression in the TCGA dataset. *HCC* hepatocellular carcinoma, *TCGA* The Cancer Genome Atlas, *GAPDH* glyceraldehyde-3-phosphate dehydrogenase, *mRNA* messenger RNA

OS, this result was validated by analyzing 370 cases from TCGA and 242 cases from GSE14520[12,13] ($p < 0.001$ for both) (Fig. 2c, d).

Associations between *RND1* expression and clinicopathological factors were then evaluated in each dataset. Notably, the low *RND1* expression group exhibited larger tumor sizes, higher levels of serum α-fetoprotein (AFP), and higher frequencies of vascular invasion than the high *RND1* expression group in our HCC cases (Table 1). Likewise, low *RND1* expression was significantly associated with clinicopathological features, indicating tumor aggressiveness in both the TCGA (electronic supplementary Table 1) and GSE14520 (electronic supplementary Table 2) datasets. Multivariate analysis revealed that *RND1* expression was an independent prognostic factor for OS (hazard ratio [HR] 2.078; $p = 0.048$) and RFS (HR 1.775; $p = 0.027$) in our HCC cases (Table 2). Similar results were obtained from multivariate analysis of OS in the TCGA (HR 1.762; $p = 0.014$) (electronic supplementary Table 3) and GSE14520 (HR 1.876; $p = 0.004$) (electronic supplementary Table 4) datasets.

Additionally, GSEA showed positive correlations between *RND1* expression and favorable prognostic gene signatures (electronic supplementary Fig. 1a, b) and an inverse correlation between *RND1* expression and an unfavorable gene signature (electronic supplementary Fig. 1c) in both the TCGA and GSE14520 datasets.

RND1 Expression was Inversely Correlated with the Ras Signaling Activity and its Knockdown Promoted Proliferation, Invasion and Chemoresistance in HCC Cells

Prior to the biological validation of these clinical findings, we performed GSEA on the TCGA dataset and estimated *RND1* expression and known oncogenic signaling pathways. GSEA showed inverse correlations between *RND1* expression and the activities of target genes in the oncogenic Ras and Myc (c-Myc) signaling pathways (electronic supplementary Fig. 2a, b), consistent with a previous report in breast cancer.[9]

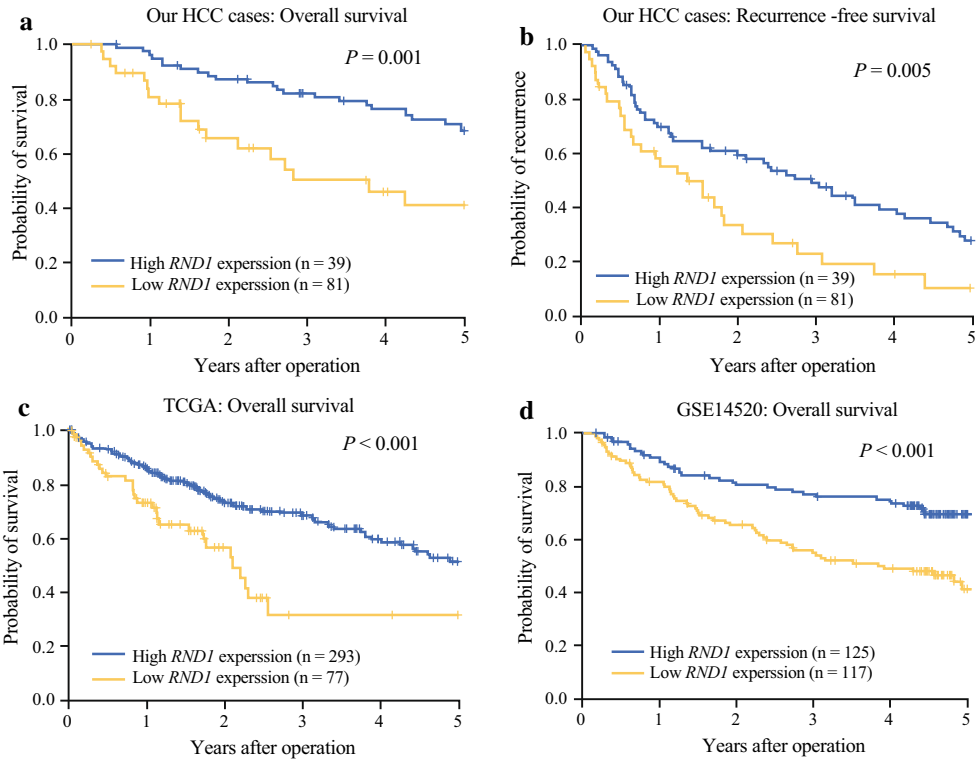


FIG. 2 Decreased *RND1* expression was associated with poor prognosis in HCC cases. **a** Overall survival in 120 patients who underwent resection for primary HCC at our hospital. **b** Recurrence-free survival in 120 patients who underwent resection for primary HCC at our hospital. **c** Overall survival in 370 patients with HCC in the TCGA dataset. **d** Overall survival in 242 patients with HCC in the GSE14520 dataset. *HCC* hepatocellular carcinoma, *TCGA* The Cancer Genome Atlas

TABLE 1 *RND1* expression and clinicopathological factors in our HCC cases ($n = 120$)

Factors	Low *RND1* expression ($n = 39$)	High *RND1* expression ($n = 81$)	p value
Age, years [mean ± SD]	64.2 ± 9.5	67.5 ± 8.6	0.067
Sex			0.265
Male	26 (66.7)	63 (77.8)	
Female	13 (33.3)	18 (22.2)	
Etiology of HBV infection			0.910
Present	10 (25.6)	20 (24.7)	
Absent	29 (74.4)	61 (75.3)	
Etiology of HCV infection			0.581
Present	24 (61.5)	54 (66.7)	
Absent	15 (38.5)	27 (33.3)	
Child–Pugh classification			0.544
A	36 (92.3)	71 (87.7)	
B or C	3 (7.7)	10 (12.3)	
AFP, ng/mL [mean ± SD]	1606.3 ± 4098.1	178.6 ± 446.6	0.036*
Maximum tumor size (cm)	5.5 ± 4.1	3.4 ± 2.4	0.008*
Number of tumors			0.556
Single	29 (74.4)	56 (69.1)	
Multiple	10 (25.6)	25 (30.9)	
Fibrous capsule formation			0.954
Present	26 (66.7)	55 (67.9)	
Absent	12 (30.8)	26 (32.1)	
NA	1 (2.6)		
Invasion to fibrous capsule			0.810
Present	22 (56.4)	45 (55.6)	
Absent	16 (41.0)	36 (44.4)	
NA	1 (2.6)		
Vascular invasion[a]			0.019*
Present	31 (79.5)	49 (60.5)	
Absent	7 (17.9)	32 (39.5)	
NA	1 (2.6)		
Biliary invasion			0.095
Present	3 (7.6)	1 (1.2)	
Absent	35 (89.7)	80 (98.8)	
NA	1 (2.6)		
Histological differentiation[b]			0.132
Well or moderately	30 (76.9)	72 (88.9)	
Poorly or undifferentiated	7 (17.9)	7 (8.6)	
NA	2 (5.1)	2 (2.5)	

Data are expressed as n (%) unless otherwise specified

[a] Invasion to portal vein or hepatic vein

[b] WHO grades 1 and 2 or Edmondson–Steiner grades 1 and 2 were classified as well-differentiated or moderately differentiated. WHO grades 3 and 4 or Edmondson–Steiner grades 3 and 4 were classified as poorly differentiated or undifferentiated

SD standard deviation, *HBV* hepatitis B virus, *HCV* hepatitis C virus, *AFP* α-fetoprotein, *NA* not available, *HCC* hepatocellular carcinoma, *WHO* World Health Organization

* Indicates statistical significance

TABLE 2 Univariate and multivariate analyses of clinicopathological factors for overall and recurrence-free survival in our HCC cases ($n = 120$)

Variable	Univariate analysis			Multivariate analysis		
	HR	95 % CI	*p* value	HR	95 % CI	*p* value
Overall survival						
Age (years)	1.008	0.974–1.048	0.672			
Sex (male/female)	1.158	0.590–2.484	0.682			
Etiology of HBV infection (present/absent)	0.945	0.425–1.892	0.879			
Etiology of HCV infection (present/absent)	1.345	0.706–2.733	0.376			
Child–Pugh classification (A/B or C)	0.748	0.339–1.975	0.525			
AFP (ng/mL)	1.00013	1.00002–1.00021	0.029*	1.00010	0.99998–1.00023	0.323
Maximum tumor size (cm)	1.163	1.058–1.264	0.003*	1.070	0.962–1.180	0.200
Number of tumors (single/multiple)	0.519	0.282–0.979	0.043*	0.623	0.317–1.269	0.187
Fibrous capsule formation (present/absent)	1.146	0.602–2.327	0.688			
Vascular invasion[a] (present/absent)	1.521	0.798	0.208			
Biliary invasion (present/absent)	7.058	1.650–20.914	0.013*	2.838	1.154–6.234	0.021*
Histological differentiation[b] (well, moderately/poorly or undifferentiated)	0.361	0.174–0.847	0.021*	0.352	0.160–0.867	0.025*
RND1 expression (low/high)	2.701	1.434–5.004	0.003*	2.078	1.005–4.174	0.048*
Recurrence-free survival						
Age (years)	1.002	1.027–0.978	0.850			
Sex (male/female)	0.996	0.621–1.658	0.987			
Etiology of HBV infection (present/absent)	0.882	0.507–1.455	0.634			
Etiology of HCV infection (present/absent)	1.060	0.677–1.697	0.801			
Child–Pugh classification (A/B or C)	0.606	0.340–1.177	1.132			
AFP (ng/mL)	1.00010	1.00002–1.00017	0.046*	1.00011	0.99995–1.00021	0.155
Maximum tumor size (cm)	1.065	0.984–1.141	0.112			
Number of tumors (single/multiple)	0.461	0.269–0.806	<0.001*	2.901	1.763–4.723	<0.001*
Fibrous capsule formation (present/absent)	1.103	0.693–1.810	0.585			
Vascular invasion[a] (present/absent)	1.404	0.889–2.276	0.148			
Biliary invasion (present/absent)	1.527	0.249–4.947	0.582			
Histological differentiation[b] (well, moderately/poorly or undifferentiated)	0.490	0.270–0.985	0.046*	0.465	0.252–0.940	0.034*
RND1 expression (low/high)	1.904	1.192–2.989	0.008*	1.775	1.069–2.886	0.027*

[a] Invasion to portal vein or hepatic vein

[b] WHO grades 1 and 2 or Edmondson–Steiner grades 1 and 2 were classified as well-differentiated or moderately differentiated. WHO grades 3 and 4 or Edmondson–Steiner grades 3 and 4 were classified as poorly differentiated or undifferentiated

HR hazard ratio, *CI* confidence interval, *HBV* hepatitis B virus, *HCV* hepatitis C virus, *AFP* α-fetoprotein, *HCC* hepatocellular carcinoma, *WHO* World Health Organization

* Indicates statistical significance

For the biological validation of the clinical findings, we performed in vitro experiments using siRNA targeting *RND1*. We used multiple HCC cell lines (HuH-7 and PLC/PRF/5) for these analyses. Our findings confirmed that cells transfected with *RND1* siRNA exhibited downregulated expression of RND1 mRNA and protein (electronic supplementary Fig. 3a, b). As a result, knockdown of *RND1* promoted cell proliferation (Fig. 3a) and increased cell invasion (Fig. 3b). Additionally, growth inhibitory assays with cisplatin were performed because GSEA also suggested that *RND1* expression was significantly related to gene signatures representing chemosensitivity to cisplatin (Fig. 3c). Actually, knockdown of *RND1* increased chemoresistance to cisplatin in PLC/PRF/5 cells (Fig. 3d).

DISCUSSION

Few studies have examined the biological role of *RND1* in cancer. Okada et al. recently reported that *RND1* functions as a negative regulator in the oncogenic Ras signaling

pathway, and *RND1* depletion enhances the malignant phenotype of breast cancer in cooperation with abnormal overexpression of *Myc*, which is a famous oncogene.[9] In contrast, Xiang et al. suggested that *RND1* may have a putative tumor-promoting role in esophageal squamous cell carcinoma.[16] Thus, owing to these contradictory findings

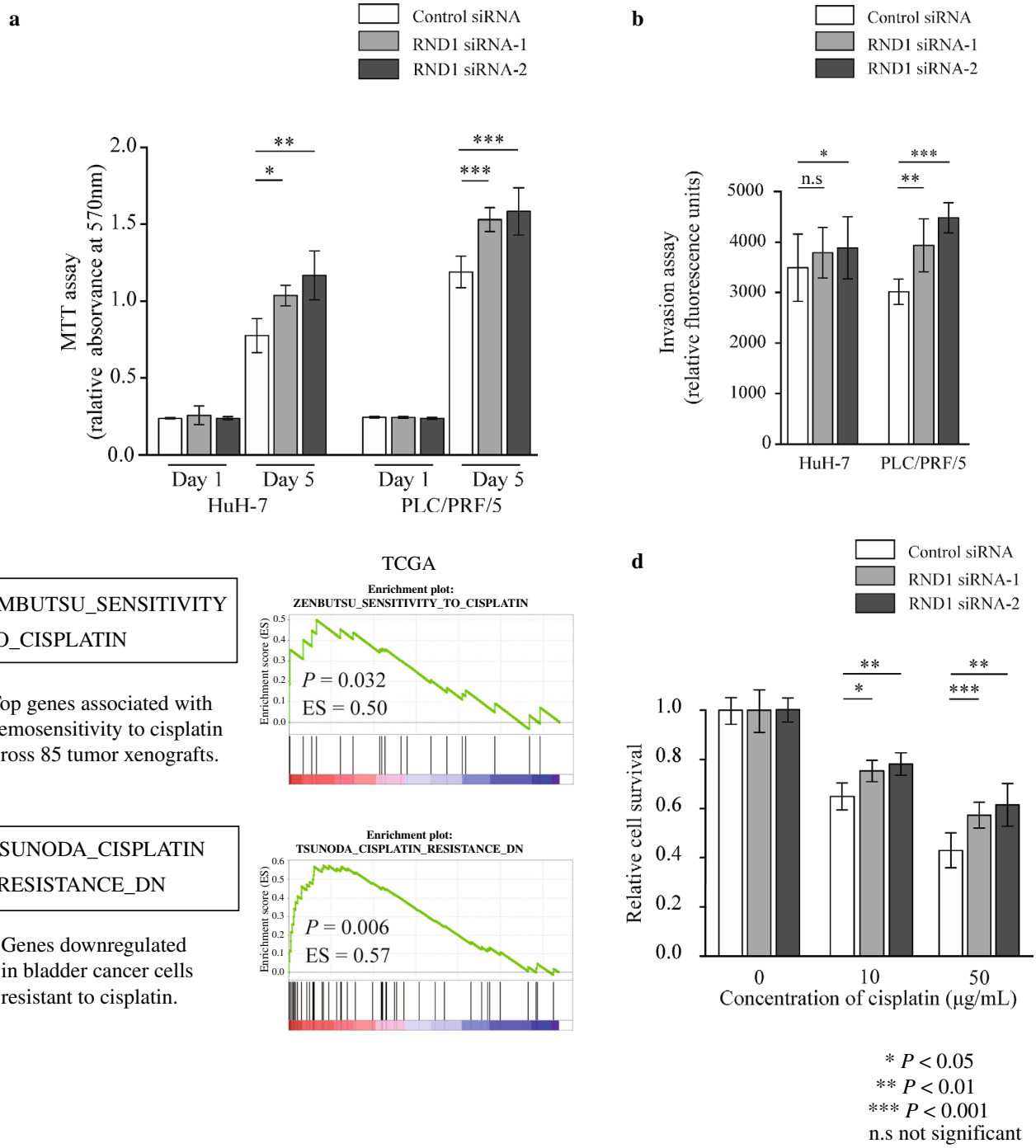


FIG. 3 Knockdown of *RND1* enhanced the malignant characteristics of HCC cells. **a** Growth ratios of HCC cells transfected with *RND1* siRNA or negative control siRNA. Knockdown of *RND1* promoted proliferation in HCC cells (* $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$). **b** Invasive capacity of HCC cells transfected with *RND1* siRNA or negative control siRNA. Knockdown of *RND1* increased invasion in HCC cells (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). **c** Gene set enrichment analysis showed a significant positive correlation between *RND1* expression and a gene signature representing sensitivity to cisplatin. **d** Survival rates in HCC cells transfected with *RND1* siRNA or negative control siRNA by growth inhibition assays with cisplatin. Knockdown of *RND1* led to increased chemoresistance to cisplatin in PLC/PRF/5 cells (** $p < 0.01$, *** $p < 0.001$). *ES* enrichment score, *n.s.* not significant, *HCC* hepatocellular carcinoma, *siRNA* small interfering RNA, *MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide*

regarding the role of *RND1* in cancer biology, the clinical impact of *RND1* expression in cancer is unclear.

In our study, we found that *RND1* expression was downregulated in HCC tissues compared with non-cancerous liver tissue, and lower expression was observed in more advanced cancers. These were consistent with the characteristics of tumor-suppressor genes,[17] thus suggesting a tumor-suppressive role of *RND1* in HCC. In addition, our data suggested that gene promoter methylation was influential in repression of *RND1* expression in HCC. Growing evidence has shown that epigenetic modifications, such as aberrant gene methylation, are a common feature in cancer genomes and are often closely related to disease progression.[18,19] Therefore, our findings implied that attenuated *RND1* expression had considerable effects on HCC progression via epigenetic alterations. Besides, such epigenetic modifications in solid cancer were of clinical interest in recent years,[20] and thus our results may offer an insight into targeted therapy for *RND1* methylation in HCC.

In survival analysis, lower expression of *RND1* predicted poorer OS in all three cohorts, and shorter RFS in our HCC cases. In all cohorts, *RND1* expression was further shown to be an independent prognostic factor for poorer OS. Additionally, GSEA results from both the TCGA and GSE14520 datasets supported these findings, providing more evidence that attenuated *RND1* expression could be a potent predictor of poor prognosis in patients with HCC.

Analysis of clinicopathological factors showed that attenuated *RND1* expression was associated with the malignant characteristics, including larger tumor sizes, higher serum AFP and increased vascular invasion as markers of tumor aggressiveness and factors predicting poor prognoses in patients with HCC.[21–23] In accordance with these results, in vitro experiments showed that knockdown of *RND1* expression promoted the proliferation and invasion of HCC cells. Ras and Myc signaling pathways are known to jointly contribute to cancer progression,[24] and enhanced activity of these pathways increases cancer cell proliferation and invasion.[25–27] Recently, Okada et al. discovered the role of *RND1* as a negative regulator of the Ras and Myc signaling pathways.[9] Based on our results showing significant inverse correlations between *RND1* expression and the Ras and Myc signaling activity by GSEA in HCC cases, our findings are consistent with these previous studies.

In our analysis, GSEA revealed that *RND1* expression was positively associated with gene signatures, representing chemosensitivity to cisplatin. Although no previous studies have demonstrated the relationships between *RND1* and chemoresistance in cancer, our in vitro experiments showed that depletion of *RND1* expression induced chemoresistance to cisplatin in HCC cells. Increased chemoresistance is a feature associated with the epithelial–mesenchymal transition (EMT) in HCC.[28] Okada et al. showed that loss of *RND1* expression induces the EMT in breast cancer cells; therefore, additional studies are needed to investigate the association between *RND1* expression and the EMT in HCC.

CONCLUSIONS

We showed that *RND1* expression decreased along with the progression of HCC, and that *RND1* downregulation by epigenetic suppression was a significant indicator of malignant potential and poor prognosis in HCC. We further validated the clinical impact of these findings by examining the biological role of *RND1* in the inhibition of proliferation, invasion, and chemoresistance, probably through suppression of the Ras signaling pathway in HCC cells. Although the mechanisms through which *RND1* influences the behaviors of other types of cancers remain unclear, we propose that *RND1* expression may be a novel biomarker for prediction of clinical outcomes, and may have therapeutic relevance in HCC.

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