Overexpression of the RD RNA binding protein in hepatitis C virus-related hepatocellular carcinoma

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Abstract. Hepatocellular carcinoma (HCC) often exhibits a poor prognosis due to metastatic spread caused by portal vein invasion (PVI). In the present study, we attempted to identify a novel therapeutic target related to PVI of HCC. Based on pooled genomic data, we identified RD RNA binding protein (RDBP), a member of the negative elongation factor (NELF) transcription elongation regulatory complex, to be preferentially overexpressed in HCC with PVI. We used quantitative reverse transcription polymerase chain reaction (RT-PCR) and immunohistochemical analyses to investigate the relationship between RDBP mRNA and protein with metastatic potential in sample sets of hepatitis C virus (HCV)-related HCC and corresponding non-HCC liver tissues. We also used the small interfering RNA technique to examine the role of RDBP in invasion and proliferation of HCC cells *in vitro*. Our data showed that both mRNA and protein levels of RDBP were significantly higher in HCC compared to non-HCC liver tissue, and that these levels were also significantly higher in HCC with PVI compared to HCC without PVI. Multivariate analysis revealed that RDBP protein levels were an independent risk factor for early intrahepatic recurrence of HCC within 2 years of surgery. Knockdown of RDBP protein significantly inhibited the proliferation and invasion of cells *in vitro*. These data demonstrate that RDBP is related to the metastatic potential of HCC, suggesting a possible candidate for prevention of HCC cell metastasis.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common fatal malignancies observed worldwide, with an annual incidence of ~600,000 deaths (1). Chronic infection with hepatitis B virus (HBV) or hepatitis C virus (HCV) is the most clearly established risk factor for HCC (2). In Japan, ~80% of HCC cases are due to chronic HCV infection, while the vast majority of the remaining cases are associated with HBV infection (3,4). Despite recent advances in patient management, the 5-year survival rate of HCC patients is as low as 26-50%, while disease-free survival is only 13-29% after curative surgery (5). This poor prognosis can be largely explained by early intrahepatic recurrence (IHR) due to metastatic spread of cancer cells via portal vein invasion (PVI) following surgery (6-8). Given this poor prognosis, there has been intense interest in identifying a strategy that may either prevent PVI, or effectively treat HCC with PVI. For this purpose, it is crucial to identify the key genes that play a central role in the development of PVI of HCC. With this in mind, we have pooled DNA microarray data of >10,000 genes in 60 primary sites of HCC (7,9). By collecting and analyzing these data, we were able to identify RD RNA binding protein (RDBP) as a unique candidate gene responsible for PVI.

The *RDBP* gene localizes to the class III region of the major histocompatibility complex (MHC) on chromosome 6, and encodes a 44-kDa nuclear protein (10,11). The encoded protein is a member of the negative elongation factor (NELF) transcription elongation regulatory complex that represses RNA polymerase II transcript elongation by acting with DRB (5,6-dichloro-1-β-D-ribofuranosylbenzimidazole) sensitivity-inducing factor (DSIF), resulting in the transcriptional pause of RNA polymerase II (12). Deletion of the RNA-recognition motif (RRM) has demonstrated that this region plays the most critical role in the transcriptional pausing among the RDBP motifs (13,14). However, to the best of our knowledge, studies examining the biological role of RDBP in human cancer cells have yet to be undertaken.

In the present study, we demonstrated for the first time that increased RDBP levels were associated with PVI and early IHR of HCV-related HCC, and that RDBP may serve as a therapeutic target for HCC with a highly malignant phenotype.

Patients and methods

Patients and tissue samples. HCV-related HCC and corresponding non-cancer liver tissues were obtained from patients who had undergone surgical resection in the Department of Digestive Surgery and Surgical Oncology, Yamaguchi University Medical Hospital between 1997 and 2007. For reverse transcrip-

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tion polymerase chain reaction (RT-PCR) analysis, tumor tissue samples and their corresponding non-tumor liver tissues were collected from 57 patients with HCC who underwent curative hepatectomy. For immunohistochemical analysis, 88 non-HCC liver and HCC tissues were collected, fixed in 10% formaldehyde solution and embedded in paraffin. Sixty-four (73%) of the 88-patient samples collected for this portion of the study had undergone curative hepatectomy. In this study, we defined IHR within 2 years of surgery as early IHR, as has been described previously (15). All samples were obtained with the patients' informed consent. The study protocol was undertaken according to the REMARK criteria (http://www.cancerdiagnosis.nci.nih.gov/assessment/progress/remark.htm.), and was approved by the Institutional Review Board for the Use of Human Subjects at Yamaguchi University School of Medicine.

Cell culture. The human HCC cell line HLE was used for all functional analyses in the present study. HLE cells were maintained in DMEM containing 10% fetal bovine serum (FBS) and antibiotics at 37˚C in a 5% humidified CO_2 atmosphere.

RNA extraction and RT-PCR. In 57 sample sets that were independent of the sample sets assigned to the DNA microarray experiment, we evaluated the reproducibility of the relationship between RDBP mRNA expression levels with PVI of HCC using semi-quantitative real-time RT-PCR. For this analysis, total RNA was extracted from 57 paired samples of frozen HCC tissue and adjacent hepatic tissue using the TRIzol method (Gibco, Carlsbad, CA, USA) according to the manufacturer's instructions. Semi-quantitative real-time RT-PCR was undertaken as described previously (16) with minor modifications. We measured mRNA levels semi-quantitatively using the D/D threshold cycle method. In addition, arginine/serine-rich splicing factor 4 (SFRS4) (17) and glyceraldehyde phosphate dehydrogenase (GAPDH) were used as the reference genes. cDNA solution corresponding to 10 ng of the initial RNA was used for PCR amplification steps that were designed using the Roche Universal Probe Library (https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp). PCR primers used were sense (5'-gcagaagaaattcaacaagctca-3') and antisense (5'-tgtgctgctgctactttgct-3') for the *RDBP* gene (NM_002904), sense (5'-gatggcagttacggttctgg-3') and antisense (5'-gccatatttatctcggccact-3') for the SFRS4 gene (NM_005626) and sense (5'-agccacatcgctcagacac-3') and antisense (5'-gcccaatacgaccaaatcc-3') for the GAPDH gene (NM_002046). The size of the PCR products for RDBP, SFRS4 and GAPDH were 76, 66 and 66 bp, respectively. The Universal Probe Library probes nos. 21, 86 and 60 (Roche Diagnostics GmbH, Mannheim, Germany) were used for measurement of RDBP, SFRS4 and GAPDH levels, respectively.

Immunohistochemical staining. Immunohistochemical staining for RDBP was performed on formalin-fixed, paraffin-embedded tissue sections using the Envision$^+$ system (Dako, Glostrup, Denmark) following the manufacturer's instructions. For antigen retrieval, slides were boiled in 0.01 M sodium citrate buffer (pH 6.0) for 20 min in a microwave oven. After blocking with 3% hydrogen peroxide (H_2O_2), the slides were incubated with rabbit polyclonal antibody 10705-AP (Proteintech Group, Chicago, IL, USA) diluted 1:80 at 4˚C overnight. The slides were then washed in buffer, incubated with biotinylated secondary antisera, and streptavidin-biotin complex/horseradish peroxidase applied. Finally, we calculated the percentage of positive cells in each lesion using a Katikati counter (http://www.vector.co.jp/soft/dl/win95/art/se347447.html). We scored the staining intensity as follows; 0, no staining; 1+, mild staining; 2+, moderate staining; 3+, intense staining. The area of staining was evaluated as follows; 0, no staining of cells in any microscopic fields; 1+, <30% of nucleus stained positive; 2+, between 30 and 60% stained positive; 3+, >60% stained positive. RDBP expression was evalueded by combined assessing of staining intensity and extension. The criteria used in this study has been widely accepted previously (18). HCC samples were categorized into weak and strong expression groups according to RDBP expression score in the nucleus. HCC with RDBP expression score equal to or <4 and those with RDBP positivity of >4 were categorized into weak and strong expression groups, respectively.

Western blot analysis. Total protein was extracted from cell lines using the protein extraction solution M-PER® Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, Rockford, IL, USA). Aliquots of total protein (50 μg for clinical samples and 30 μg for cell lines) were electrophoresed on 10-20% gradient precast gels (System Instruments Co., Tokyo, Japan) and electroblotted onto pure nitrocellulose membranes [iBlot Transfer Stack, Mini (Nitrocellulose), Invitrogen, Carlsbad, CA USA].

RDBP protein was then detected using rabbit polyclonal antibody 10705-AP (Proteintech Group) diluted 1:500. RDBP protein levels were normalized to the level of GAPDH (SC 25778, Santa Cruz Biotechnology, Santa Cruz, USA). Blots were developed with horseradish peroxidase-linked anti-rabbit immunoglobulin SC 2004 (Santa Cruz Biotechnology) diluted 1:7500. Supersignal West Pico Trial Kit Reagents (Thermo Fisher Scientific) were used to detect antigen-antibody reactions.

siRNA transfection. Small interfering RNA (siRNA) for RDBP was custom synthesized by Qiagen, (Qiagen, Hilden, Germany). Target sequences were as follows: si RDBP-1 5'-CGGGATCGGGATCGAGATCGA-3' and si RDBP-2 5'-CAAGGTGGTGTCAAACGCTCA-3'. Non-specific control siRNA was obtained from B-Bridge International (S10C-0600, Cupertino, CA). SiRNA to RDBP1,2 or non-specific siRNA (both 100 pmol/ml) was transfected using Lipofectamine 2000 reagent (Invitrogen) in serum-free Opti-MEM I (Invitrogen) according to the manufacturer's instructions and as described previously (16).

Proliferation assay. To evaluate cell survival and proliferation, CellTiter 96RAQueous One Solution Cell Proliferation Assay (MTS assay, Promega, Madison, WI, USA) was performed. Cells ($2x10^4$) were seeded into the wells of 96-well plates after the transfection of siRNA and incubated at 37˚C in a humidified atmosphere with 5% CO_2. At the appropriate time, 20 μl of CellTiter 96RAQueous one solution was added to each well and the plates incubated for a further 4 h at 37˚C. The optical density was then measured at 490 nm using a 96-well plate reader. Triplicate wells were analyzed in each assay.

Invasion assay. HLE cells transfected either with RDBP siRNA or non-specific control siRNA were cultured in DMEM containing 5% FBS. The cells were then harvested by trypsinization, washed in DMEM without serum and suspended in

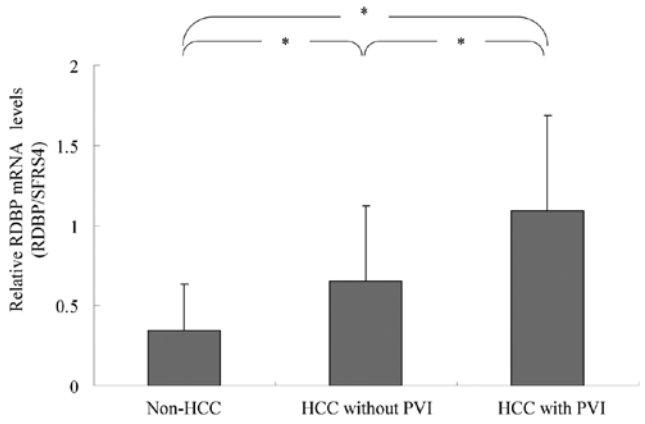

Figure 1. *RDBP* mRNA levels relative to the internal control SFRS4 determined by quantitative RT-PCR (box and whisker plot). *RDBP* mRNA levels were significantly higher in HCC (n=57) than in non-HCC liver tissues (n=57). Note that *RDBP* mRNA levels were significantly higher in HCC with PVI (n=18) than in HCC without PVI (n=39). *P<0.05.

DMEM at $4x10^4$ cells/ml. Prior to the preparation of the cell suspension, a dried layer of Matrigel matrix (Becton-Dickinson Biosciences, San Jose, CA, USA) was rehydrated with DMEM for 2 h at room temperature. DMEM (0.75 ml) containing 2% FBS was then added to the lower chambers of a 24-well Matrigel invasion chamber, and 0.5 ml ($2x10^4$ cells) of cell suspension added to each insert of the upper chamber. The plates and inserts were incubated for 24 h at 37˚C. After incubation, the cells invading through the Matrigel were fixed and stained with hematoxylin. As a control, uncoated polycarbonate membrane (Becton-Dickinson) was used instead of the Matrigel chamber. The number of cells in each membrane was also counted under a microscope (magnification x50) using a Katikati counter. Triplicate wells were analyzed for each assay.

Statistical analysis. Student's t-test and Mann-Whitney U test were used to evaluate differences between two or more continuous variables. Fisher's exact test or χ^2 test was used to evaluate the differences between discontinuous variables. We carried out multivariate analysis to assess independent factors for early IHR using the multiple logistic regression models. All statistical analyses were performed using SPSS 11.0J (SPSS, Inc., Chicago, IL, USA) software. P<0.05 was considered significant.

Results

Identification of RDBP as a candidate gene procedure. Using pooled DNA array data, we identified 40 genes demonstrating expression levels that were higher by >4-fold in HCC tissues when compared with non-cancer liver tissues. We next filtered 18 genes for which expression levels were significantly higher in HCC with PVI than in HCC without PVI. We subsequently ranked the 18 genes according to the magnitude of their mRNA levels and then examined immunohistochemically their protein levels in several sample sets of HCC and corresponding non-HCC liver tissues. Our preliminary examination demonstrated that RDBP expression levels were abundant in HCC tissues and were significantly higher in HCC with PVI than in HCC without PVI.

RDBP mRNA expression. RDBP mRNA expression was significantly higher in HCC without PVI (n=39) than in non-HCC liver samples (n=57, 0.65±0.47 vs. 0.34±0.29 (mean ± SD), P<0.01). RDBP mRNA levels were also significantly higher in HCC with PVI (n=18) than in HCC without PVI (n=39, 1.09±0.60 vs. 0.65±0.47, P<0.01, Fig. 1).

RDBP protein expression. To evaluate whether RDBP protein levels were associated with the metastatic potential of HCC, we performed immunohistochemical staining for RDBP on 88 sample sets of HCC and corresponding non-HCC liver tissues, and 10 normal liver tissues in which normal liver function was confirmed by a blood test. RDBP protein was predominantly expressed in the nucleus of HCC cells (Fig. 2). RDBP protein

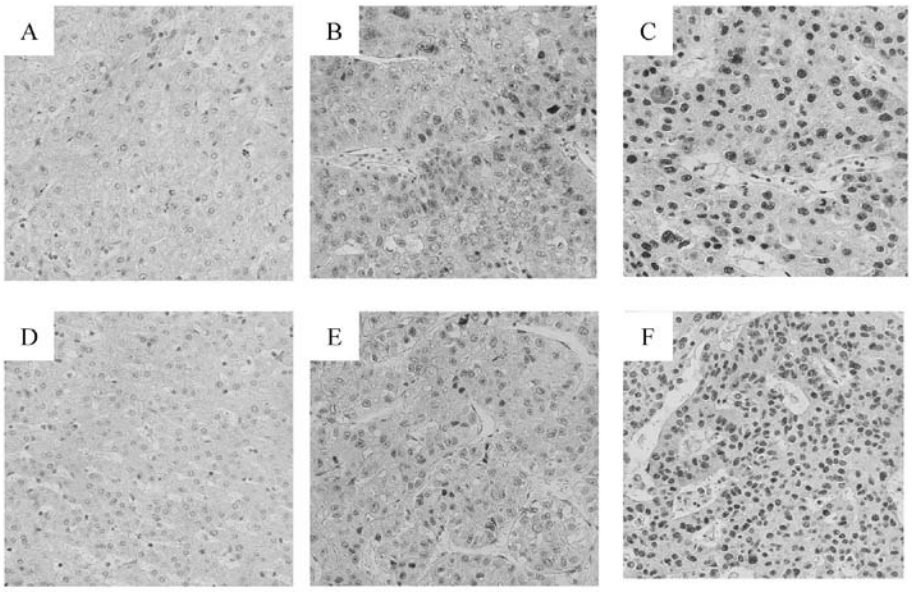

Figure 2. Immunohistochemical staining of RDBP protein in the human liver. Non-HCC liver (A and D) and HCCs without PVI (B and E) demonstrate low or no expression of RDBP protein. In contrast, HCCs with PVI (C and F) show markedly high levels of RDBP protein. Notably, RDBP protein was strongly expressed in the majority of HCC with PVI (C and F), but was weakly expressed in HCC without PVI (B and E). Original magnification x200.

Table I. RDBP protein expression in human liver samples.

RDBP protein expression	HCC patient		Normal (n=10)	P-value
	HCC (n=88)	Non-tumor (n=88)		
Negative	4 (4.5%)	70 (79.5%)	10	<0.01
Weak	33 (37.5%)	16 (18.2%)	0	
Strong	51 (58.0%)	2 (2.3%)	0	

Table II. Clinicopathologic findings of HCC in relation to RDBP expression.

Clinicopathologic factor	RDBP expression		P-value
	High (N=51)	Low (N=37)	
Age	68.0±8.4	67.8±7.2	0.90
Gender			0.44
Male	38	29	
Female	13	8	
Tumor size			0.08
<3 cm	24	11	
≥3 cm	27	26	
Degree of differentiation			<0.01
Well	5	12	
Moderate	35	24	
Poor	11	1	
Portal vein invasion (PVI)			<0.01
Negative	31	33	
Positive	20	4	
TNM stage			0.28
I+II	21	19	
III+IV	30	18	
Early intrahepatic recurrence	High (N=40)	Low (N=24)	0.02
Negative	15	16	
Positive	25	8	

was expressed in the majority of HCC tissues (84/88, 95%). In contrast, staining for RDBP protein was observed in only 20% of the non-HCC liver tissues (Fig. 2A and D). According to the protein expression score, we determined that staining for RDBP was weak in 33 (37.5%) and strong in 51 (58.0%) of the HCC tissues. Staining for RDBP was weak in 16 (18.2%) and strong in 2 (2.3%) of the non-HCC liver tissues. Thus, RDBP protein levels were found to be significantly higher in HCC tissues than in non-HCC liver tissues (P<0.0001 by Fisher's exact test). No staining for RDBP protein was observed in the 10 control liver tissues (Fig. 2 and Table I).

Table III. Independent risk factors for early intrahepatic recurrence (IHR).

Factors	Regression coeffcient	Standard error	Risk ratio (95% CI)	P-value
RDBP group	4.928	0.542	3.333 (1.151-9.650)	0.026

Correlation of RDBP expression with clinicopathological features. RDBP protein level was positively associated with PVI and de-differentiation grade (P<0.05 for both, Table II). In contrast, RDBP protein level was not associated with other factors such as age, gender, tumor size and pathological TNM classification of malignant tumors (TNM) stages.

RDBP overexpression as an import predictive marker for early intrahepatic recurrence. RDBP protein levels were significantly higher in HCC with early IHR (n=33) than in HCC without early IHR (n=31, Table II). To identify independent risk factors for early IHR, 5 variables including primary tumor number, tumor size, tumor differentiation, portal invasion, and RDBP expression were entered into a multivariate regression analysis. The logistic regression analysis selected only one variable, RDBP expression group (P=0.026) as risk factors for early IHR (Table III).

RDBP function in cell proliferation. To assess whether RDBP is essential for growth or survival of HCC cells, we used siRNA technology to reduce RDBP levels. When we transfected siRNAs against RDBP (RDBP si-RNA1 and si-RNA2) into HLE cells, we found that HLE-siRNA1 and HLE-siRNA2 cells showed markedly lower levels at RDBP mRNA and protein levels than the si-control cells (Fig. 3A and B). Down-regulation of RDBP expression by siRNA also led to a significant growth inhibition and a significant reduction in the cellular activity of HLE cells when compared with the si-control cells (Fig. 3C and D).

RDBP function in cell invasion. Cell invasion was investigated using the Matrigel cell invasion chamber. Down-regulation of RDBP expression by siRNA led to a decrease in the number of invasive cells when compared with the si-control cells (Fig. 3E), thus independently suggesting that RDBP plays an important role in the invasive ability of HCC cells.

Discussion

It is well established that vessel invasion (VI) including PVI, tumor number and tumor size are representative risk factors of a poor prognosis for HCC patients undergoing hepatic surgery (19). Among these factors, PVI is considered to be a hallmark of

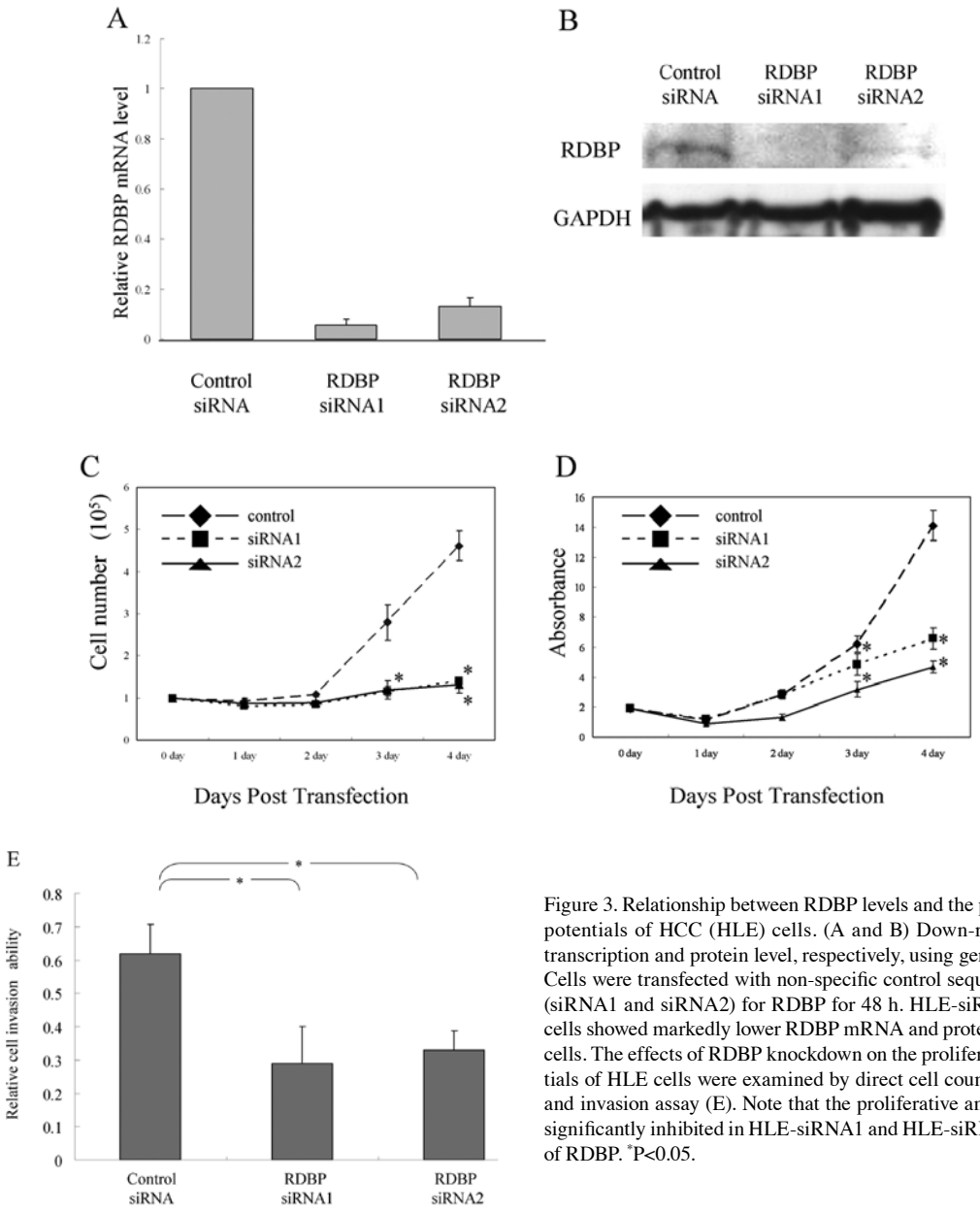

Figure 3. Relationship between RDBP levels and the proliferative and invasive potentials of HCC (HLE) cells. (A and B) Down-regulation of RDBP at a transcription and protein level, respectively, using gene targeting with siRNA. Cells were transfected with non-specific control sequence or siRNAs specific (siRNA1 and siRNA2) for RDBP for 48 h. HLE-siRNA1 and HLE-siRNA2 cells showed markedly lower RDBP mRNA and protein levels than the control cells. The effects of RDBP knockdown on the proliferative and invasive potentials of HLE cells were examined by direct cell counting (C), MTS assay (D) and invasion assay (E). Note that the proliferative and invasive potentials are significantly inhibited in HLE-siRNA1 and HLE-siRNA2 cells with low levels of RDBP. *P<0.05.

the intrahepatic spread of HCC cells and of poor outcome (20). It is therefore crucial for hepatologists to indentify key genes or proteins that play a central role in the PVI process of HCC. To this end, the present study focused on PVI-related genes and successfully identified a novel PVI-related gene, RDBP, in HCC from thousands of genes on the DNA array without any bias. We found that mRNA levels of RDBP were significantly higher in HCV-related HCC with PVI than in those without PVI, and that the encoded protein was increased in parallel to the progression of poorly differentiated HCC. Strikingly, the protein level of RDBP was an independent risk factor for early IHR within 2 years of surgery.

Several DNA microarray studies have identified VI-related genes in a manner similar to our study. Chen *et al* (21) identified 91 genes for which expression levels were significantly correlated with the presence or absence of VI. Okabe *et al* (22) identified 151 VI-related genes including 110 ESTs, *RHOC* and two small GTPase-related genes known as *ARHGAP8* and *ARHGEF6*. A study by Tsunedomi *et al* (23) focused on moderately differentiated HCV-related HCC to minimize the bias of gene selection, as VI is not detected in well-differentiated HCC, but is frequently observed in moderately or less well-differentiated HCC, and identified 35 VI-related genes. Tanaka *et al* (24) identified 28 VI-related genes including AURKB using microarray clustering based on macroscopic findings for VI. Thus, although much effort has been devoted to identification of VI-related genes, there are, so far, few genes or gene products that can be applied to the daily clinical use of HCC treatment. One possible deficiency may be explained by the fact that gene levels are not always related to those of the encoded protein. In this regard, RDBP identified in the present study is fascinating from the viewpoint of a target molecule specific for HCC as its protein is abundant in HCC, but not in non-HCC liver tissues and in various non-malignant epithelia (data not shown).

It is generally accepted that HCC recurrence is a complicated process. There are at least three modes of postoperative

recurrence (25) including early and late IHR that appear in the remnant liver, and extra-hepatic recurrence that appears in distant tissues and organs. In this study, we found that RDBP protein level was positively associated with early IHR, but not late IHR. Additionally, our multivariate analysis revealed that RDBP was an independent risk factor for early IHR. These results appear to be reasonable, considering that late IHR is caused by *de novo* hepatocarcinogenesis such as multicentric occurrence (25), which can be affected by the background liver status in chronic liver disease, but not tumor factors. In contrast, most early IHRs are due to intrahepatic metastasis of cancer cells and are detected in 30-50% of patients within two years of surgery, limiting the potential for surgical cure of HCC (7,26,27). Taken together, we strongly suggest that overexpression of RDBP may account for the highly metastatic potential of HCC.

In the present study, our experimental finding showed that knockdown of RDBP inhibited the invasive potential of HCC cells, supporting our clinical finding that RDBP was overexpressed in HCC with PVI. However, the precise role of RDBP in the metastatic process of cancer cells remains unclear. An elegant study by Narita *et al* (28) showed that although knockdown of RDBP/NELF-E inhibited the proliferation of HeLa cells, there were no significant difference in the cell cycle distribution between the control and knockdown cells. Consistent with this, we confirmed both the growth inhibitory effects and the lack of change in cell cycle pattern in HCC cells by RDBP knockdown (data not shown). Narita *et al* (28) revealed that knockdown of RDBP/NELF-E promoted the transcription of replication-dependent histone genes. It remains unclear as to how histone protein family is linked to invasion potentials of cancer cells. Therefore, confirmation of this finding is beyond the scope of the present study. Intriguingly, recent studies showed that COBRA1/NELF-B and TH1/NELF-C/D were decreased in advanced breast cancer and that knockdown of the two genes enhanced motility of the breast cancer cells (29,30). Thus, NELF family proteins may have opposite effects in cancer progression in a tissue-dependent manner. Further examination is required to gain insight into this concept.

The precise mechanisms underlying the up-regulation of RDBP at a transcriptional level in HCC with PVI remain unknown. One possible explanation is that this gene may be epigenetically regulated via CpG islands at the promoter region. To test this possibility, we undertook a preliminary examination to investigate the epigenetic status of the promoter region of the *RDBP* gene in four paired sets of HCC and corresponding non-HCC liver tissue using methylation-specific PCR (MSP). Our MSP analysis revealed that the *RDBP* gene remained unmethylated in all of the samples tested (data not shown), raising the possibility that RDBP was up-regulated by mechanisms other than promoter methylation.

More recently, enhancement of HCC patient prognosis using specific targeted agents such as Sorafenib has been reported (31,32). Although Sorafenib inhibits proliferation and angiogenesis in HCC, it is unclear whether it directly inhibits tumor invasion and metastasis. In this regard, and given the finding that RDBP is closely associated with the proliferation and invasive potentials of HCC cells, we suggest that RDBP may prove useful as a potential therapeutic target for HCC, especially in advanced HCC with PVI.

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