Identification of *ID2* associated with invasion of hepatitis C virus-related hepatocellular carcinoma by gene expression profile

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Abstract. Portal vein invasion (PVI) is a hallmark of metastatic potential of hepatocellular carcinoma (HCC) and is frequently found at a stage of moderately differentiated HCC. To identify genes involved in PVI of HCC associated with hepatitis C virus (HCV), we performed a comprehensive analysis of 12,600 genes in 35 moderately differentiated HCV-related HCCs by DNA microarray. Our supervised learning method identified 35 genes involved in PVI. Among the 35 identified genes, we focused on the inhibitor of DNA binding 2 *(ID2)*, because it encodes a liver-rich dominant-negative helix-loop-helix protein. The microarray results for *ID2* were reproduced by quantitative real-time reverse transcription (QRT)-PCR and Western blot analyses. In an independent set of HCV-related HCCs (n=28) and HCV-unrelated HCCs (n=14), our QRT-PCR showed that decrease in ID2 mRNA levels were associated with PVI in HCV-related HCC but not HCV-unrelated HCC. In conclusion, our results strongly suggest that *ID2* plays an important role in PVI process of HCV-related HCC.

Introduction

Hepatocellular carcinoma (HCC) is one of the most lethal malignancies (1,2). The poor prognosis of HCC can be explained largely by the high rate of intrahepatic recurrences attributable to intrahepatic dissemination of tumor cells (3). Among many factors responsible for intrahepatic recurrence, venous invasion, particularly portal vein invasion (PVI), is one of the most relevant pathologic factors (4). Identification of key genes involved in PVI will allow for improved treatment of HCC.

Since its initial application to clinical science (5), DNA microarray technology has served as a promising tool for elucidation of molecular features of HCC (6,7). Therefore, it may be useful for identifying key genes involved in PVI. Okabe *et al* (8) showed that small GTPase-related genes such as *RhoGAP8* and *ARHGEF6* are preferentially down-regulated in HCC with PVI. However, factors such as sample bias may lead to differing results among microarray studies (9). In particular, infection with specific hepatitis viruses can affect HCC gene profiles (10,11). In this regard, the study used cohorts comprising HCC patients infected with different hepatitis viruses (8). To extend the previous finding, it is necessary to profile genes involved in PVI in HCC according to infection with hepatitis B or C virus (HBV or HCV).

We recently identified genes related to dedifferentiation of HCV-related HCC (12). Sample rearrangement by molecular profiling showed that moderately differentiated HCCs without PVI were clustered close to well-differentiated HCC, and moderately differentiated HCCs with PVI were clustered close to poorly differentiated HCC. Thus, PVI is induced in moderately differentiated HCC. This result is supported by previous clinicopathologic findings (13). The present study focused on identification of PVI-related genes in HCV-associated, moderately differentiated HCC. We identified 35 PVI-related genes, including inhibitor of DNA binding 2 *(ID2)*, which encodes a dominant-negative helix-loop-helix (HLH) protein and is abundant in human liver (14). To our knowledge, this is the first identification of the *ID2* in PVI in HCV-related HCC.

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Key words: hepatocellular carcinoma, portal vein invasion, hepatitis C virus, inhibitor of DNA binding 2

Table I. Clinicopathological features of the 77 HCCs used in this study.

	Samples for DNA microarray	Independent samples for QRT-PCR	
	Moderately differentiated HCV-related HCC (n=35)	Moderately differentiated HCV-related HCC (n=28)	Moderately differentiated HCV-unrelated HCC (n=14)
Age (years)			
<50	1	0	2
≥50	34	28	12
Sex			
Male	24	22	12
Female	11	6	2
PVI			
-	21	20	7
+	14	8	7
Size (cm)			
<3	10	8	5
≥3	25	20	9
Stage			
I	12	9	4
I	18	17	8
III	5	2	2

PVI, portal vein invasion; stage, TNM stage of UICC (2002).

Materials and methods

Samples for DNA microarray study. We previously pooled oligonucleotide array datasets (huU95A DNA Chips®; Affymetrix, Santa Clara, CA) of 76 HCCs (12). Briefly, the specimens obtained at surgery were immediately frozen in liquid nitrogen for RNA extraction. Total RNA was extracted with Sepasol-RNAI (Nacalai Tesque, Tokyo, Japan) and purified with the RNeasy Mini Kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. The quality of extracted RNA was confirmed by the appearance of characteristic 28S and 18S rRNA fragments on agarose gels (data not shown). From the above-mentioned datasets, we selected data for 12,600 genes of 35 moderately differentiated HCCs with HCV infection. Clinicopathological features of the 35 HCCs are shown in Table I.

Written informed consent was previously obtained from all patients. The study protocol was approved by the Institutional Review Board for Human Use at Yamaguchi University School of Medicine.

Screening of PVI-related genes by DNA microarray. PVI was histologically diagnosed in 14 (40%) of the 35 HCCs. We used the Fisher ratio (3,10,12) to evaluate the potentials of the selected genes to discriminate between 14 HCCs with PVI and 21 HCCs without PVI. Of 12,600 genes on the DNA microarray, we used 3,559 genes with expression levels >40 (arbitrary units by Affymetrix) because we confirmed that genes with expression levels of ≤40 were biologically insignificant in our previous experiment with this-type DNA microarray (15). The 3,559 genes were ranked in order of decreasing magnitude of the Fisher ratio. To determine the number of genes to be considered, a random permutation test was performed as described previously (10). From distribution of the Fisher ratios based on randomized data, all genes that passed the random permutation test ($P<0.004$) were selected. Differences in expression levels of 35 genes with Fisher ratios >1.10 between HCCs with PVI and HCCs without PVI were statistically significant. The false discovery rate (FDR), i.e., the percentage of genes selected by chance, was calculated as described previously (12) and was 0.08%, validating the gene selection procedure.

Validation of DNA microarray data for ID2 by quantitative real-time RT-PCR (QRT-PCR). To investigate the reproducibility of ID2 mRNA levels identified by DNA microarray analysis, we used RNA stocks from 22 HCV-related HCC samples used in the DNA microarray. Quantitative real-time RT-PCR (QRT-PCR) was performed according to a previously described method (16) with minor modifications. In brief, real-time PCR amplification (LightCycler System Version 3; Roche Diagnostics, Mannheim, Germany) was performed with 10 pmol of each primer sets (ID2-S 5'-GAACTGC AGTTTTAATGGGCAGGAGATGC-3' and ID2-AS 5'-GGA AAGCTTCAGTGCAAGGTAAGTGATGG-3' for *ID2*; and GAPDH-S 5'-CCTTCATTGACCTCAAC-3' and GAPDH-AS 5'-AGTTGTCATGGATGACC-3' for GAPDH) and cDNA equivalent to 10 ng initial RNA. The PCR mixture included 2 μl Master Mix (LightCycler FastStart DNA Master SYBR Green I; Roche Diagnostics) and 4 mM $MgCl_2$. Amplification was performed according to a three-step cycle procedure consisting of 40 cycles of denaturation at 95˚C for 10 sec, annealing at 55˚C for 10 sec, and extension at 72˚C for 20 sec. PCR products were quantified with a Lumi-Imager F1 (Roche Diagnostics) and analyzed with LightCycler software (Roche Diagnostics).

We measured quantitatively mRNA levels for *ID2* genes using crossing point (CP) and PCR efficiency (E) as described previously (17). The CP is the number of PCR cycles when maximal acceleration of the fluorescence increase is reached. The investigated transcripts showed PCR efficiencies of 1.69 for *GAPDH* and 1.75 for *ID2*. Results, expressed as target gene expression relative to the GAPDH, termed 'N_{target}', were determined by the formula: $N_{target} = E_{target}^{[40\ (\text{maximal cycle number})-CPtarget]}/E_{GAPDH}^{[40-CPGAPDH]}$. The specificity of the products was documented with a gel electrophoresis and analysis of the melting temperature.

Validation of ID2 protein levels by Western blot analysis. Six HCC tissues, which were subjected to the DNA microarray study, were homogenized in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 165 mM NaCl, 1 mM PMSF, 10 mM NaF, 10 mM EDTA, 1 mM vanadate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 0.1% NP-40. Protein (15 μg) from total cell lysates was fractionated by SDS-PAGE and transferred electrophoretically to PVDF membranes with a Hoefer electrotransfer system (Amersham Biosciences,

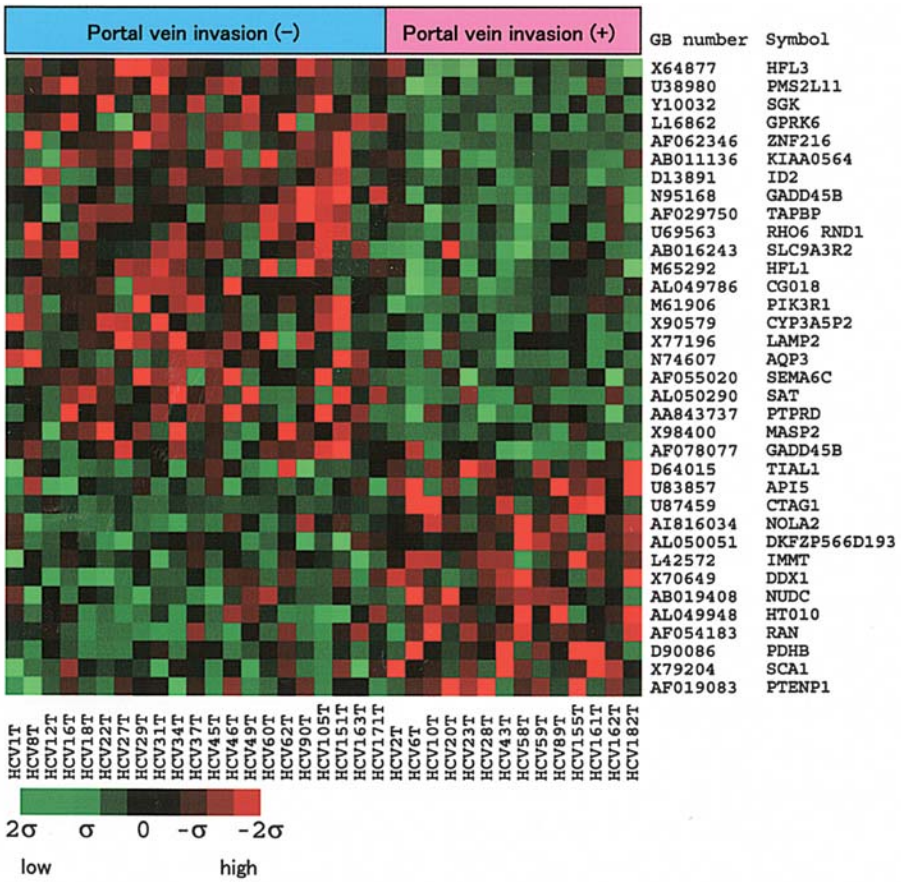

Figure 1. The 35 genes involved in PVI in HCV-related HCC. The top 35 genes were selected by the Fisher ratio and are listed by GenBank accession number and Entrez gene symbol (http://www.ncbi.nih.gov/entrez/query.fcgi?db=gene).

Piscataway, NJ, USA). To detect ID2 and GAPDH protein, membranes were incubated with rabbit polyclonal ID2 (C-20, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) or GAPDH (FL-335, Santa Cruz Biotechnology, Inc.) antibody. Detection was performed with a secondary horseradish peroxidase-conjugated anti-rabbit antibody and the ECL chemiluminescence system (Amersham Biosciences). The mean band densities were determined using NIH Image 1.62 software, and calculated as levels relative to GAPDH.

Assessment of ID2 mRNA levels in an independent set of HCC by QRT-PCR. To investigate the relation among ID2 mRNA levels, PVI, and HCV infection pattern, we examined ID2 mRNA levels in newly enrolled 42 HCCs (Table I) by means of QRT-PCR. Among the 42 patients, 28 were positive for HCV antibody and the remaining 14 were negative for HCV antibody. PVI was proven pathologically in 8 (28.6%) of the 28 HCV-related HCCs and in 7 (50%) of the 14 HCV-unrelated HCCs. Total RNA was extracted from HCC tissues with TRIzol® reagent (Invitrogen Corp., Carlsbad, CA, USA), and 1 μg total RNA was reverse-transcribed with 5 U avian myeloblastosis virus reverse transcriptase (Takara Bio Inc., Shiga, Japan) at 45˚C for 30 min. QRT-PCR procedure was performed as described above. Written informed consent was also obtained from all the 42 patients.

Statistical analysis. Data were presented as mean ± standard deviation. Significant differences between 2 groups were evaluated by Student's t-test or Mann-Whitney U test. Significant differences between 3 or more groups were evaluated by ANOVA with Scheffe's or Dunnett's test. The Pearson correlation coefficients were calculated for the expression levels of ID2 between DNA microarray and QRT-PCR analyses. Calculations were performed with SPSS II software for Windows (SPSS Inc., Chicago, IL). $P<0.05$ was considered statistically significant.

Results

With our gene selection procedure, 35 genes were identified with expression levels that differed significantly between 14 tumors with PVI and 21 tumors without PVI of the 35 HCV-related HCCs (Fig. 1 and Table II). Among these genes, expression of 13 was up-regulated and that of 22 was down-regulated in HCCs with PVI compared to HCCs without PVI. The 35 PVI-related genes included the gene responsible for apoptosis and stress response (*SGK*, *API5*, and *GADD45B*), cell cycle and cell proliferation (*RAN* and *NUDC*), oncogenesis (*DDX1*), signal transduction (*RHO6*), and immune response (*TIAL1* and *TAPBP*). Thus, the selected genes showed a variety of biological function.

Among the 35 PVI-related genes, we focused on *ID2*, one of HLH-type transcriptional modulators, because it was shown to encode a liver-rich protein (14). Moreover, another statistical analysis showed that ID2 mRNA levels were significantly lower in HCCs with PVI than in those without

Table II. Top-35 discriminatory genes in HCC with and without PVI.

Fisher ratio	GB no.	Description	Symbol	Locus	Function
12 genes up-regulated in HCCs with PVI in comparison with HCCs without PVI					
1.496	U83857	Apoptosis inhibitor 5	*API5*	11p12-q12	Inhibition of apoptosis
1.357	U87459	Cancer/testis antigen 1	*CTAG1*	Xq28	Tumor antigen
1.342	AI816034	Nuclear protein family A, member 2	*NOLA2*	5q35.3	rRNA processing and midification
1.282	AL050051		DKFZP566D193	3q21.1	Unknown
1.248	L42572	Inner membrane protein, mitochondrial (mitofilin)	*IMMT*	2p11.2	It interacts with cytoskeletal components
1.159	X70649	DEAD/H box polypeptide	*DDX1*	2p24	RNA helicase/oncogenesis
1.141	AB019408	Nuclear distribution gene C homologue	*NUDC*	1p35-p34	Cell proliferation
1.141	AL049948	Uncharacterized hypothalamus protein HT010	*HT010*	2q32.2	Unclear
1.139	AF054183	RAN, member RAS oncogene family	*RAN*	6p21	Mitosis/cell cycle control
1.132	D90086	Pyruvate dehydrogenase (lipoamide) ß	*PDHB*	3p21.1-p14	Glucose metabolism
1.118	X79204	Spinocerebellar ataxia 1	*SCA1*	6p23	Neuronal transmission
1.102	AF019083	Phosphate and tensin homologue, pseudogene 1	*PTENP1*	9p21	Unclear
23 genes down-regulated in HCCs with PVI in comparison with HCCs without PVI					
2.021	X64877	H factor-like 3	*HFL3*	1q31-q32.1	Complement activation
1.828	U38980	Postmeiotic segregation increased 2-like 11	*PMS2L11*	7q	Unclear
1.643	Y10032	Seru/glucocorticoid regulated kinase	*SGK*	6q23	Stress response/ sodium transport
1.587	L16862	G protein-coupled receptor kinase	*GPRK6*	5q35	Signal transduction
1.542	AF062346	Zinc finger protein 216	*ZNF216*	9q13-q21	Nucleic acid-binding protein
1.536	D64015	TIA1 cytotoxic granule-associated RNA binding protein-like 1	*TIAL1*	10q	Immune response
1.477	AB011136		*KIAA0564*	13q13.3	Unknown
1.406	D13891	Inhibitor of DNA binding 2	*ID2*	2p25	It may negatively regulate cell differentiation
1.368	N95168	GADD45B growth arrest and DNA-damage-inducible, ß	*GADD45B*	19p13.3	Stress response/apoptosis
1.345	AF029750	TAP binding protein (tapasin)	*TAPBP*	6p21.3	Immune response/ antigen processing
1.248	U69563	GTP-binding protein RHO6	*RHO6*	12q12-q13	Cytoskelton and cell adhesion
1.234	AB016243	Solute carrier family 9, isoform 3 regulatory factor 2	*SLC9A3R2*	16p13.3	Sodium/hydrogen exchanger
1.234	M65292	H factor-like 1	*HFL1*	1q32	Complement activation
1.226	AL049786	Hypothetical gene CG018	*CG018*	13q12-q13	Unknown
1.218	M61906	Phosphoinositide-3-kinase, regulatory subunit, polypeptide 1	*PIK3R1*	5q12-q13	Signal transduction/ metabolic actions of insulin
1.211	X90579	Cytochrome P450, subfamily IIIA, polypeptide 5 pseudogene 2	*CYP3A5P2*	7q21.3-q22	Detoxification system
1.198	X77196	Lysosomal-associatiated membrane protein 2	*LAMP2*	Xq24	Protection, maintenance, and adhesion of the lysosome
1.192	N74607	Aquaporin 3	*AQP3*	9p13	Molecule transport/ water channel protein
1.186	AF055020	Semaphorin Y	*SEMA6C*	1q21.2	Neural regeneration
1.181	AL050290	Spermidine/spermin N1-acetyltransferase	*SAT*	Xp22.1	Polyamine catabolism
1.154	AA843737	Protein tyrosine phosphatase, reseptor type, D	*PTPRD*	9p23-q24.3	Signal transduction
1.149	X98400	Mannan-binding lectin serine protease 2	*MASP2*	1p36.3-p36.2	Complement activation
1.140	AF078077	GADD45B growth arrest and DNA-damage-inducible, ß	*GADD45B*	19p13.3	Stress response/apoptosis

GB number and Symbol of each gene were obtained from Entrez Gene (http://www.ncbi.nih.gov/entrez/query.fcgi?db=gene).

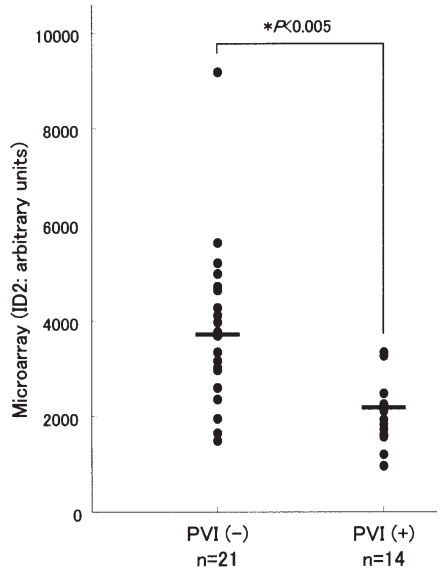

Figure 2. The relation between levels of ID2 mRNA and PVI in HCV-related HCCs. The data for ID2 expression was extracted from DNA microarray dataset of 35 moderately differentiated HCV-related HCCs (HCV-antibody positive). ID2 mRNA levels (mean ± SD) were 3744.7±1710.3 vs 2114.3±762.8 in HCCs without PVI (n=21) and HCCs with PVI (n=14), respectively. ID2 mRNA levels were significantly lower in HCCs with PVI than in those without (*P<0.005 by Student's t-test).

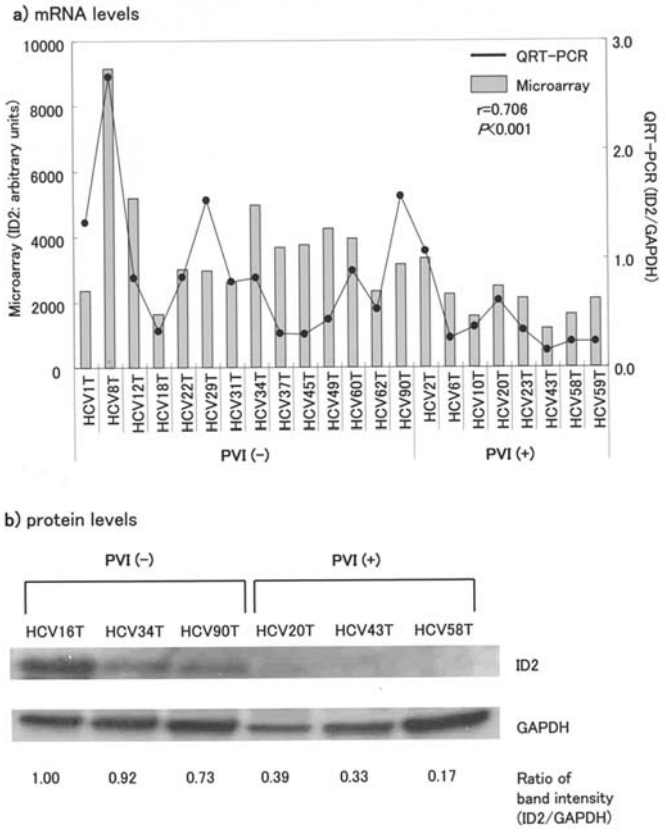

Figure 3. Validation of microarray data for ID2 by quantitative real-time RT-PCR and Western blot analyses. (a) ID2 mRNA levels measured by quantitative real-time RT-PCR (QRT-PCR) were calculated relative to those of GAPDH. Note the correlation between the RT-PCR data and the microarray data (r=0.71, P<0.001). (b) Western blot analysis showed significantly lower levels of ID2 protein in HCCs with PVI than in those without PVI (mean ± SD: 0.30±0.11 vs 0.88±0.14, P<0.005). The band intensity was measured by using NIH image 1.62 and calculated as levels relative to that of GAPDH.

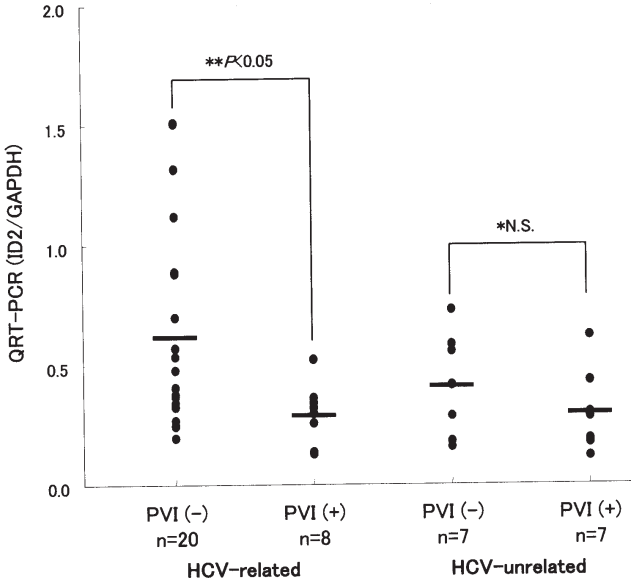

Figure 4. The relation between levels of ID2 mRNA and PVI in HCV-related and HCV-unrelated HCCs. The data for ID2 expression was extracted from newly enrolled 42 HCCs. We examined the relation between ID2 levels and PVI in two cohorts of 28 moderately differentiated HCV-related HCCs (HCV-Ab positive) and 14 moderately differentiated HCV-unrelated HCCs (HCV-Ab negative). In 42 HCCs, ID2 mRNA levels (mean ± SD) were 0.63±0.43, 0.29±0.13, 0.41±0.22, and 0.30±0.17 in HCV-related HCCs without PVI (n=21), HCV-related HCCs with PVI (n=14), HCV-unrelated HCCs without PVI (n=7), and HCV-unrelated HCCs with PVI (n=7), respectively. Note that the relation of ID2 levels to PVI is specific to HCV-related HCC. *Student's t-test; **Mann-Whitney's U test; N.S., not significant.

PVI (P<0.005 by Student's t-test) (Fig. 2). There was a strong association between ID2 mRNA levels in the microarray data and those in QRT-PCR (r=0.706, P<0.001) (Fig. 3a). Western blot analysis showed that ID2 protein levels were also significantly lower in HCCs with PVI than in those without PVI (P<0.005 by Student's t-test) (Fig. 3b).

To clarify whether ID2 mRNA levels were associated with PVI in an independent cohort and whether the decrease was specific to HCV-related HCC, we performed QRT-PCR analysis in newly enrolled 42 moderately differentiated HCCs (28 were HCV-related HCCs and 14 were HCV-unrelated HCCs). In 28 HCV-related HCCs, ID2 mRNA levels (mean ± SD) were 0.29±0.13 and 0.63±0.43 in tumors with PVI and those without PVI, respectively. Thus, ID2 mRNA levels were significantly lower in HCV-related HCCs with PVI than in those without PVI (P<0.05 by Mann-Whitney U test) (Fig. 4). In contrast, there was no statistical difference between ID2 mRNA levels in HCV-unrelated HCCs with PVI and those without PVI (Fig. 4). These results show that decrease in ID2 mRNA levels are associated with PVI in HCV-related HCC, but not HCV-unrelated HCC.

Discussion

DNA microarray technology allows for analysis of simultaneous expression of thousands of genes and has opened new avenues in molecular medicine (5). Studies with DNA microarray have identified many molecular targets linked to various aspects of HCC (8,10-12,18-23). Notably, several investigators have used this technology to classify HCC on

the basis of clinicopathological behavior such as metastatic potential (23-26). PVI is critical for intrahepatic metastasis of HCC (4); therefore, better understanding of the molecular basis of PVI would help to improve the poor outcome of HCC. However, there have been no DNA microarray studies investigating genes specific for PVI in HCV-related HCC. In the present study, we identified 35 genes linked to PVI in HCV-related HCC without bias among thousands of genes. The most striking finding of our study is that decreased levels of *ID2* gene are associated with PVI of HCV-related HCCs but not HCV-unrelated HCCs.

ID proteins are HLH proteins without the basic DNA-binding domain that negatively regulate transcription of target genes by dimerizing with other basic HLH proteins (25,27,28). Many studies have revealed the function of ID proteins as regulators of cell growth and differentiation (27,29-31). Among *ID* family genes, *ID2* is abundant in human liver (14), indicating a significant role of ID2 in hepatocyte differentiation. Our previous study showed that ID2 mRNA levels were lower in HCV-related well-differentiated HCC than in non-tumorous liver, suggesting a role of ID2 in hepatocarcinogenesis induced by HCV (12). Given our present finding that levels of *ID2* were significantly decreased in moderately differentiated HCC with PVI compared to those in moderately differentiated HCC without PVI, down-regulation of *ID2* appears to be linked to tumor progression of HCV-related HCC. A recent report shows that ID2 protein levels decrease in parallel with HCC progression (32). Unfortunately, that study (32) used a cohort consisting of HBV- and HCV-related HCCs. Our present study not only corroborates the finding (32) but also shows that the role of *ID2* is specific for progression of HCV-related HCC.

Other PVI-related genes identified in our study included the gene responsible for apoptosis and stress response (*SGK*, *API5*, and *GADD45B*), cell cycle and cell proliferation (*RAN* and *NUDC*), oncogenesis (*DDX1*), signal transduction (*RHO6*), and immune response (*TIAL1* and *TAPBP*). Our previous study showed that *SGK* was significantly down-regulated in HCC with PVI and was related to intrahepatic recurrence of HCC (24). The levels of *SGK* transcript are altered in hepatoma cells in response to osmotic changes or cell volume changes (33); however, it remains unclear how *SGK* is related to PVI. *GADD45B* was found to be down-regulated in HCC by the promoter hypermethylation (34). *RHO6*, one of *Rh*o GTPase family genes, regulates negatively cell adhesion and the down-regulation of *Rho* family genes in HCC with PVI was reported in previous microarray study (8). Down-regulation of immune response-related genes (*TIAL1* and *TAPBP*) in HCC with PVI is reasonable, because many DNA microarray studies have proposed the significant role of immune response-related genes in HCC progression (8,22,24,26,35). Thus, these genes identified here can be candidates of molecular targets in the treatment of tumor with PVI. Deeper investigation is warranted to disclose their precise action in PVI of HCC.

Our identification of *ID2* as a PVI-related gene in HCV-related HCC was reproduced by the finding that decreased levels of ID2 mRNA were associated with PVI in independent HCC samples of large size. Genome-wide approaches have attracted a great deal of attention in the field of HCC research. Many studies (6-12,18-26) have identified genes linked to several clinicopathologic features of HCC. However, there was little overlap between the genes identified in the various DNA microarray studies, and interpretations of the data remain controversial. These inconsistencies may be related closely to differences in sample populations (9). In this regard, our present study adjusted tumor differentiation and hepatitis virus type to avoid a gene-selection bias caused by different sample backgrounds. As a result, we successfully identified *ID2* gene in relation to PVI in HCV-related HCC. Our present result also indicates the potential of *ID2* as a therapeutic target in the progression of HCV-related HCC but not of HCV-unrelated HCC. Elucidation of the mechanism underlying down-regulation of *ID2* in parallel with HCV-related HCC progression may lead to the use of *ID2* as a marker for dedifferentiation of HCV-related HCC.

Acknowledgements

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