Decreased ID2 Promotes Metastatic Potentials of Hepatocellular Carcinoma by Altering Secretion of Vascular Endothelial Growth Factor

Ryouichi Tsunedomi,1,2 Norio Iizuka,1,3 Takao Tamesa,1 Kazuhiko Sakamoto,1 Takashi Hamaguchi,1 Hideaki Somura,1 Mamoru Yamada,4 and Masaaki Oka1

Abstract

Purpose: We aimed to explore the molecular and biological functions of Inhibitor of DNA binding/differentiation 2 (ID2), which was found to be responsible for portal vein invasion of hepatocellular carcinoma (HCC).

Experimental Design: We measured ID2 mRNA levels in 92 HCC patients by real-time reverse transcription-PCR and examined the relation to clinicopathologic features. To clarify the precise roles of ID2, we did in vitro analysis with expression vectors and small interfering RNAs. Effects of ID2 on cell invasive potential and expression of vascular endothelial growth factor (VEGF) and hypoxia-inducible factor-1α were analyzed by Matrigel-coated invasion chamber, ELISA, and Western blot analysis, respectively.

Results: ID2 mRNA level correlated inversely with portal vein invasion (P < 0.001), tumor-node-metastasis stage (P < 0.001), tumor size (P < 0.001), and early intrahepatic recurrence (P < 0.05). When limited to a cohort of hepatitis C virus–related HCCs, patients with low levels of ID2 had significantly shorter disease-free survival time than those with high levels of ID2. Invasive potential of cells transfected with ID2 expression vector was lower than that of empty vector–transfected cells. Cells overexpressing ID2 also showed decreased VEGF secretion and hypoxia-inducible factor-1α protein levels. The results of ID2 knockdown experiments were opposite to those of ID2 overexpression experiments.

Conclusions: On the basis of our clinical and in vitro data, we suggest that ID2 plays a significant role in the metastatic process during progression of HCC. This action might be explained, at least in part, by altered cell mobility due to decreased secretion of VEGF.

Hepatocellular carcinoma (HCC) is the sixth most common malignant tumor worldwide and accounted for 5.7% of new cancer cases in 2002 (1). HCC is caused mainly by chronic liver inflammation due to hepatitis B virus, hepatitis C virus (HCV), alcohol abuse, or hemochromatosis (2). Despite

resection with curative intent and recent advances in treatments, the clinical course of HCC is variable, and recurrence occurs in a large number of patients after surgery. The poor prognosis can be explained largely by the high rate of intrahepatic recurrence (IHR) attributable to intrahepatic dissemination of tumor cells (3). Among the many factors responsible for IHR, venous invasion, particularly portal vein invasion (PVI), is one of the most significant pathologic factors (4). Thus, identification of key genes involved in PVI may improve therapies for HCC.

Hepatitis B virus and HCV contribute differently to the molecular pathogenesis of HCC (2). This concept was supported by our previous genome-wide studies (5, 6). We examined HCV-related HCC and identified a gene, inhibitor of DNA binding/differentiation 2 (ID2), whose levels were significantly lower in HCV-related, well-differentiated HCC than in HCV-related liver disease (7). More recently, we found that ID2 is a PVI-related gene specific for HCV-related HCC (8). ID proteins (ID1-ID4), which belong to a helix-loop-helix family of proteins, act as dominant-negative inhibitors of basic helix-loop-helix transcription factors by forming heterodimers (9–11). ID proteins are involved in proliferation processes, differentiation, development, senescence, and angiogenesis (12–16) that are linked to various malignancies (17–32). Interestingly, it is likely that ID1 and ID2 function differently in progression of breast cancer (21, 25, 29). In the case of HCC,
expression of ID1 is increased at early stages of hepatocarcinogenesis (31), and high ID1 expression increases the metastatic potential of HCC (32). It is known that ID2 is expressed at high levels in both human adult and fetal liver tissues (33) but that expression of ID2 decreases in more advanced HCC (34). However, it remains unclear how ID2 is linked to the malignant potential of HCC.

In the present study, we confirmed the clinicopathologic significance of ID2 expression in a large number of HCC-related HCCs by semiquantitative real-time reverse transcription-PCR (RT-PCR) analysis. We then examined the effect of ID2 expression on invasive potential and vascular endothelial growth factor (VEGF) secretion by HCC cells. This is the first study to describe the precise role of ID2 in the metastatic process during progression of HCC.

Materials and Methods

Samples. Samples were obtained with informed consent from 92 patients who underwent curative hepatectomy for HCC between May 1997 and November 2004 in the Department of Digestive Surgery and Surgical Oncology, Yamaguchi University Graduate School of Medicine, Japan. The study protocol was approved by the Institutional Review Board for Human Use at Yamaguchi University Graduate School of Medicine. Clinicopathologic features of the 92 HCCs in this study are described in Table 1. Among the 92 HCC patients examined, 53 were positive for HCV antibody, 25 were positive for hepatitis B virus surface antigen, and the remaining 14 were negative for both (Table 1). We defined the latter two-types of HCC as HCV-unrelated HCC. None of the 92 patients had hemochromatosis in their liver. Patients were defined as habitual drinkers when they had a history of continuous intake of >30 g of ethanol per day. All patients were followed up after hepatectomy as reported previously (35). In the present study, we defined IHR within 1 year after surgery as early IHR, most of which are due to intrahepatic spread of cancer cells (35).

HCC cell lines. Human HCC cell lines Hep3B, HepG2, HLE, HuH-6, and HuH-7 were used in this study. These cell lines were purchased from the Health Science Research Resources Bank. Cells were cultured in DMEM (Nissui Pharmaceutical) containing 10% heat-inactivated fetal bovine serum (Invitrogen) supplemented with penicillin (100 units/mL), streptomycin (100 μg/mL), and sodium bicarbonate (1.5 g/L) at 37 °C in 5% CO2 in air.

Plasmid construction and transfection of vector encoding human ID2. Full-length human ID2 cDNA was subcloned into pcDNA3.1(-) (Invitrogen) to generate pID2. HLE cells were transfected with pID2 and Lipofectamine 2000 according to the manufacturer’s recommendations. Two clones were isolated and designated HLE/pID2-I and HLE/pID2-II. HLE cells transfected with empty vector, pcDNA3.1(-), were used as a control.

Gene knockdown. Transfection complexes were prepared by mixing each small interfering RNAs [siRNA, siID2-I, targeting ID2 (5’-GAAAAGGACAGCAAGGGATT-3’); siID2-II, targeting ID2 (5’-CGAU- GACGCCGUAUCAACAGG-3’); siVEGF-I, targeting VEGF (5’- GCACAUGUGUACAGGAUAU-3’); and siVEGF-II, targeting VEGF (5’-AGCAUGAUUGAUUGUGUAU-3’) from Nippon EGT; or siCont negative control siRNA (S10C-0600; B-Bridge International)] and Lipofectamine 2000 reagent (Invitrogen) in serum-free Opti-MEM I. An equal volume of cells suspended in Opti-MEM I supplemented with 5% fetal bovine serum was added (final: 1 × 10⁵ cells, 30 nmol/L siRNA, 0.15% Lipofectamine 2000 reagent, and 2.5% fetal bovine serum in Opti-MEM I).

Table 1. Relations between ID2 mRNA levels and clinicopathologic features in HCC

<table>
<thead>
<tr>
<th>PVI</th>
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<th>ID2 mRNA level*</th>
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<tr>
<td></td>
<td></td>
<td>All HCC (n = 92)</td>
<td>HCV-related HCC (n = 53)</td>
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<tr>
<td>Stage</td>
<td></td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.005</td>
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<tr>
<td></td>
<td>-</td>
<td>1.19 ± 0.83 (n = 61)</td>
<td>1.33 ± 0.87 (n = 38)</td>
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<tr>
<td></td>
<td>+</td>
<td>0.62 ± 0.51 (n = 31)</td>
<td>0.66 ± 0.55 (n = 15)</td>
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<tr>
<td>Tumor size (cm)</td>
<td></td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.005</td>
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<tr>
<td></td>
<td>&lt; 3</td>
<td>1.34 ± 0.98 (n = 34)</td>
<td>1.45 ± 0.97 (n = 22)</td>
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<td></td>
<td>≥ 3</td>
<td>0.80 ± 0.57 (n = 58)</td>
<td>0.91 ± 0.65 (n = 33)</td>
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<tr>
<td>Primary tumor type</td>
<td></td>
<td>P &lt; 0.05</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td></td>
<td>Single</td>
<td>1.21 ± 0.90 (n = 50)</td>
<td>1.43 ± 0.97 (n = 26)</td>
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<tr>
<td></td>
<td>Multiple</td>
<td>0.75 ± 0.54 (n = 42)</td>
<td>1.02 ± 0.75 (n = 24)</td>
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<td>Age (y)</td>
<td></td>
<td>N.S.</td>
<td>N.S.</td>
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<tr>
<td></td>
<td>&lt; 60</td>
<td>1.11 ± 0.87 (n = 51)</td>
<td>1.25 ± 0.92 (n = 29)</td>
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<tr>
<td></td>
<td>≥ 60</td>
<td>0.86 ± 0.65 (n = 41)</td>
<td>1.02 ± 0.75 (n = 24)</td>
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<tr>
<td>Sex</td>
<td></td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>0.95 ± 0.72 (n = 23)</td>
<td>1.22 ± 0.85 (n = 5)</td>
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<tr>
<td></td>
<td>Female</td>
<td>0.96 ± 0.76 (n = 69)</td>
<td>1.11 ± 0.88 (n = 39)</td>
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<tr>
<td>Alcohol abuse (g/d)</td>
<td></td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>&lt; 30</td>
<td>1.12 ± 0.86 (n = 23)</td>
<td>1.23 ± 0.77 (n = 14)</td>
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<tr>
<td></td>
<td>≥ 30</td>
<td>0.97 ± 0.89 (n = 30)</td>
<td>1.14 ± 1.08 (n = 18)</td>
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Abbreviations: Stage, tumor-node-metastasis stage of Unio Internationale Contra Cancrum (Italian; 2002). Early IHR, IHR within 1 year after surgery. N.S., not significant.

*Represented values are ID2 mRNA levels (mean ± SD) quantified by semi-qRT-PCR as described in the Materials and Methods section; the values were subsequently normalized as the mean ratio of the average value from all 92 samples to equal a value of 1.
Semi-quantitative real-time RT-PCR. Semi-quantitative real-time RT-PCR was done as described previously (8) with minor modifications. We measured mRNA levels semi-quantitatively by ΔΔ threshold cycle method, and GAPDH was used as a reference gene. The values are expressed as relative to appropriate control (average of all 92 samples, control plasmid–, or siRNA-transfected samples).

Western blot analysis. Cells were homogenized in M-PER (Pierce). Fifteen micrograms of protein was fractionated by SDS-PAGE (10-20% gradient acrylamide) and transferred electrophoretically to nitrocellulose membranes. To detect ID2, HIF1α, and glycolaldehyde-3-phosphate dehydrogenase proteins, rabbit polyclonal anti-ID2 (C-20; Santa Cruz Biotechnology), anti-HIF1α (SC10790; Santa Cruz Biotechnology), and anti–glycolaldehyde-3-phosphate dehydrogenase (Fl-335; Santa Cruz Biotechnology) antibodies were used, respectively. Detection was done with a horseradish peroxidase–conjugated anti-rabbit antibody and ECL chemiluminescence system (GE Healthcare). The mean band densities were determined with Image J software5 and calculated as levels relative to glycolaldehyde-3-phosphate dehydrogenase.

Scratch assay. Confluent cell monolayers were scratched by manually scraping the cells with a 20 to 200 μl pipette tip. Culture medium was then replaced with fresh medium containing 10 ng/ml mitomycin C (Sigma-Aldrich) to inhibit cell proliferation, and movement of cells into the denuded area was assessed by microscopy (IX71; Olympus) 24 h later. If necessary, 25 ng/ml recombinant human (rh) VEGF121 or rhVEGF165 (R&D Systems) was added to the above medium. Cell-free areas were measured with Image J software. Triplet determinations were analyzed in each assay, and assays were repeated at least twice.

Cell invasion assay. Matrigel-coated filter inserts sized to fit into 24-well plates were purchased from Becton Dickinson. Cells were resuspended in serum-free DMEM and added to the upper compartment. DMEM containing 2% fetal bovine serum was added to the lower compartment. If necessary, 25 ng/ml rhVEGF121 or rhVEGF165 were added to the medium. After 22 h (HLE derivatives) or 48 h (HuH-7 derivatives) of incubation, cells that invaded through the Matrigel were counted under a photonic microscope (IX71; Olympus). As a control, uncoated polycarbonate membrane (Becton Dickinson) was used instead of the Matrigel chamber. Triplicate wells were analyzed in each assay, and assays were repeated at least twice.

Quantification of secreted VEGF. When cells reached 80% confluence, DMEM supplemented with 10% fetal bovine serum was replaced with serum-free DMEM, and cells were incubated for 24 h. Media were collected and concentrated with centrifugal filters (Millipore). VEGF secreted into the medium was measured by ELISA with a Human VEGF Immunoassay (R&D Systems). Triplicate wells were analyzed in each assay, and assays were repeated at least twice.

Statistical analysis. Data are presented as mean ± SD. Significant differences between two groups were evaluated by Student’s t test or Mann-Whitney U test. Significant differences between three or more groups were evaluated by ANOVA with Scheffe’s or Dunnett’s test. Disease-free survival (DFS) and statistical significance were analyzed by log-rank test. We carried out multivariate analysis to assess independent factors for early IHR in the 92 HCC samples using the stepwise logistic regression model. Calculations were done with Dr. SPSS II software (SPSS). A P value of <0.05 was considered statistically significant.

Results

Correlation between tumor ID2 mRNA level and clinicopathologic features. Our previous array-based gene profile data (7, 8) showed that ID2 mRNA levels decrease in parallel with HCC progression (Supplementary Fig. S1). Our semi-quantitative real-time RT-PCR analysis showed that levels of ID2 mRNA correlated inversely with presence of PVI (P < 0.001), tumor-node-metastasis stage (P < 0.001), tumor size (P < 0.001), and presence of early IHR (P < 0.05) in the 92 HCCs (Table 1). In the 53 HCV-related HCCs, ID2 mRNA levels correlated with PVI, tumor-node-metastasis stage, tumor size, and early IHR; however, ID2 mRNA levels correlated only with tumor-node-metastasis stage in the 39 HCV-unrelated HCCs (Table 1). No correlation between alcohol abuse and tumor ID2 mRNA levels was observed in HCV-related and HCV-unrelated HCCs. Multivariate analysis revealed that, among the 9 factors, tumor ID2 mRNA levels and HCV infection were independent risk factors for early IHR in the 92 HCCs (Supplementary Table S1).

Impact of tumor ID2 mRNA levels on DFS. Our semi-quantitative real-time RT-PCR analysis showed that ID2 mRNA levels in HCC were significantly lower than those in the noncancerous liver (P < 0.001; Fig. 1A). When the receiver operating characteristic curve for discrimination between HCC and noncancerous liver was calculated (Fig. 1B), the maximum area under the curve was 0.89 (95% confidence interval, 0.83-0.95) for ID2. On the basis of the receiver operating characteristic curve, in discriminating HCC and noncancerous liver, the optimal cutoff value of ID2 mRNA levels was determined to be 1.71, which corresponded to a skewed position on the HCC histogram (Supplementary Fig. S2). On the basis of the cutoff value, DFS was calculated with the

Fig. 1. Kaplan-Meier curves for DFS of patients with high and low ID2 mRNA levels after surgery. A, box and whiskers plot for ID2 mRNA levels in noncancerous liver and HCC tissues determined by semi-quantitative real-time RT-PCR. *, P < 0.001. B, receiver operating characteristic curve for ID2. The optimal cutoff point of 1.71 was determined by selecting the point on the receiver operating characteristic curve that maximized both sensitivity and specificity. C, in our total patient population (n = 92), patients with low ID2 mRNA levels tended to have a shorter DFS time (P = 0.080). D, in a subset of 53 HCV-related HCC patients, patients with low ID2 mRNA levels had a significantly shorter DFS time (P = 0.020). Broken lines, patients with high ID2 mRNA levels; thin lines, patients with low ID2 mRNA levels.

5 http://rsb.info.nih.gov/ij/
Kaplan-Meier method (Fig. 1C and D). Patients with low ID2 mRNA expression ($n = 77$) tended to have shorter DFS ($P = 0.06$) than those with high ID2 mRNA expression ($n = 15$). Notably, correlation of ID2 mRNA level with DFS rate was found in the cohort of HCV-related HCCs ($n = 53$; $P = 0.02$).

**Effect of up-regulation and down-regulation of ID2 on cell growth.** From the five cell lines, we selected HLE cells, which express low levels of ID2 mRNA, and HuH-7 cells, which express high levels of ID2 mRNA for gene-targeting studies. Overexpression of ID2 in stably transfected HLE lines was confirmed at the mRNA and protein levels by semiquantitative real-time RT-PCR and Western blot analysis, respectively (Fig. 2). ID2 mRNA levels of HLE/pID2-I and HLE/pID2-II cells were ~7.5- and 12.7-fold higher, respectively, than those of HLE cells transfected with empty vector, pcDNA3.1 ($P < 0.001$ for both). HuH-7 cells transfected with siRNAs targeting ID2 (siID2-I and siID2-II) showed ~7.1- and 13.8-fold lower ID2 mRNA levels than HuH-7 cells transfected with control siRNA ($P < 0.001$ for both). Western blot analysis detected higher levels of ID2 protein in HLE/pID2-I and HLE/pID2-II cells than in HLE cells transfected with pcDNA3.1. ID2 protein expression was lower in HuH-7 cells transfected with siRNAs for ID2 than in HuH-7 cells transfected with control siRNA.

5-Bromo-2'-deoxyuridine assay revealed that overexpression of ID2 had no significant effect on proliferation of HLE cells but that underexpression of ID2 decreased proliferation of HuH-7 cells ($P < 0.001$; Supplementary Fig. S3).

**Migration and invasive potential of cells with overexpression or knockdown of ID2.** We used a scratch assay to examine the ability of HLE and HuH-7 derivatives to migrate under conditions in which cell proliferation was abolished by mitomycin C. HLE/pID2-I and HLE/pID2-II cells moved into the cell-free area more slowly than HLE/pcDNA3.1 cells (Fig. 3A). In contrast, ID2 knockdown in HuH-7 cells increased cell motility (Fig. 3B).

The invasive potentials of HLE/pID2-I and HLE/pID2-II cells were significantly lower (~1.6- and 1.8-fold) than that of HLE/pcDNA3.1 cells ($P < 0.001$; Fig. 3C). Conversely, ID2 knockdown by two independent siRNAs increased the invasive potential of HuH-7 cells ($P < 0.05$; Fig. 3D).

**VEGF and HIF1α expression in HLE and HuH-7 derivatives.** Significantly lower levels of VEGF were secreted from HLE/pID2-I and HLE/pID2-II cells than from HLE/pcDNA3.1 cells ($P < 0.001$; Fig. 4A). Conversely, ID2-knockdown HuH-7 cells showed significantly higher VEGF secretion than HuH-7/siCont cells ($P < 0.01$; Fig. 4B). Western blot analyses of conditioned medium from cells with altered ID2 expression showed similar results (data not shown). The decreased migration and invasive phenotypes of ID2 overexpressing cells were rescued by administration of rhVEGF (Fig. 3A and C). Conversely, inhibition of VEGF by siRNAs diminished the ID2-knockdown-induced increase in cell migration activity (Fig. 3B and D). Western blot analysis revealed that the intensity of a band corresponding to a known VEGF regulator, hypoxia-inducible factor-1α (HIF1α), was opposite to that of ID2 (Fig. 2B and D).

We also found the decreased levels of HIF1α mRNA in the ID2-overexpressing cells; however, we did not found its increased levels in both the two ID2-knockdown cells (Supplementary Fig. S4).

**Discussion**

The present study highlighted ID2, an helix-loop-helix transcription factor, as a signature molecule responsible for PVI of HCV-related HCC. In a large cohort of HCC, our current semiquantitative real-time RT-PCR analysis reproduced our previous finding that tumor ID2 mRNA levels are correlated with PVI (8) and provided a novel finding that tumor ID2 mRNA levels can be an independent risk factor for early IHR due to metastasis and for poor prognosis of HCC. The relation of tumor ID2 mRNA level to highly malignant potential was specific for HCV-related HCC but not HCV-unrelated HCC. Taken together with our previous finding (8), our present data suggest that ID2 may play an important role in the metastatic process of HCV-related HCC. This prompted us to examine the biological function of ID2 in HCC metastasis in greater detail because HCV-related HCC is not only increasing worldwide but is also a predominant type of HCC in Japan (1, 5).

Our gene-targeting *in vitro* analyses revealed that altered ID2 expression can significantly affect migration and invasive potential of HCC cells, supporting our clinical data that ID2 mRNA levels correlate with high metastatic potential of HCC. To gain deeper insights into this *in vitro* finding, we next searched for molecules that affect HCC invasion. We measured VEGF and matrix metalloproteinase 1 levels because the significance of these molecules in the metastasis of HCC has been reported (36–39). We found an inverse correlation between ID2 expression and VEGF secretion *in vitro*. Furthermore, the
modulation of migration and invasive abilities by ID2 expression was depending on VEGF level. VEGF promotes cell invasion and migration in vitro (40–42), and it plays a central role in the invasive and metastatic potentials of various tumors, including HCC (36, 37). Our preliminary study showed that expression of MMP1 mRNA is remarkably lower in ID2-overexpressing HCC cells than in control cells. However, knockdown of ID2 failed to show a relation between ID2 levels and matrix metalloproteinase 1 levels (data not shown). Taken together, our results suggest that ID2 may regulate the malignant phenotype of HCC by modulating VEGF but not matrix metalloproteinase 1.

ID2 proteins can bind transcription factors, such as basic helix-loop-helix and Ets proteins, to regulate transcriptional activity (9–11). Possible partners of ID2 may include transcription factors that regulate cell proliferation, migration, and invasion potentials as mentioned above. Our present results show that in HLE and HuH-7 cells, HIF1α protein level is associated inversely with ID2 protein level but that HIF1α mRNA level is not always associated inversely with ID2 protein level. It is known that HIF1α is rapidly targeted to degradation by the proteasome under normal oxygen conditions, but it is stabilized and permits activation of many hypoxia-related genes, including VEGF and MMPs, under hypoxic conditions (45). Given that our in vitro experiment was done under normal oxygen conditions and ID2 did not regulate transcription of HIF1α, it is reasonable to assume that a small amount of ID2 can stabilize HIF1α protein, and that increased HIF1α protein induces transcription of VEGF in HCC cells. This lack of transcriptional regulation of HIF1α by ID2 is supported by our previous finding that the HIF1α gene was not identified in a screen for genes related to PVI of HCC (8).
More recently, it was reported that ID1 enhances the stability and activity of HIF1α by decreasing the association of HIF1α with von Hippel-Lindau because von Hippel-Lindau is a subunit of ubiquitin ligase complex directing HIF1α degradation (46). Thus, ID1 and ID2 have opposite effects on HIF1α stability, suggesting the possibility of mutual regulation of IDs. An elegant study by Lee et al. (32) showed that high ID1 expression increases the metastatic potential of HCC by enhancing VEGF expression through stabilization of HIF1α protein. In contrast, we found that high ID2 decreases the metastatic potential of HCC by inhibiting VEGF expression through destabilization of HIF1α protein. Thus, it is possible that ID1 and ID2 have opposite effects on HCC metastasis. Lee et al. (32) used a cohort consisting mostly of hepatitis B virus–related HCC. In contrast, our cohort consisted of many HCV-related HCCs. Differences in hepatitis virus type can have large effects on genetic and phenotypic patterns of HCC (5); therefore, it is impossible to compare directly our present finding with those of Lee et al. (32). Interestingly, it was reported that HCV infection leads to HIF1α stabilization and consequent VEGF stimulation (47), suggesting that HCV itself may be related to HIF1α-mediated regulation of VEGF in HCC. Because ID2 mRNA levels are down-regulated during early-stage HCC caused by HCV infection (7), ID2 might participate in the VEGF modulation caused by HCV infection. Further studies are needed to identify the partners of ID2 and to clarify the relations between ID2 and HCV-related proteins.

In conclusion, we found that ID2 expression is associated with a risk of HCC progression. The possible association of ID2 with VEGF secretion via HIF1α level highlights the importance of this association in PVI and indicates that ID2 may be a potential diagnostic and therapeutic target for HCC, especially HCV-related HCC.

References
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