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Silencing of *Hint1*, a novel tumor suppressor gene, by promoter hypermethylation in hepatocellular carcinoma

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Abstract

The Hint1 protein, a member of the histidine triad (HIT) family, is highly conserved in diverse species and ubiquitously expressed in mammalian tissues. Previous studies in mice provided evidence that *Hint1* may be haplosufficient with respect to its function as a tumor suppressor. In the present study, we investigated the aberrant methylation of *Hint1* and explored possible relationships between aberrant methylation and clinicopathological features in hepatocellular carcinoma (HCC). Hypermethylation of *Hint1* was evaluated by the methylation specific PCR (MSP) method in 40 patients with HCC (tumor and paired adjacent non-tumor tissues) from Taiwan, 22 cases of normal liver tissue (14 from Taiwan and 8 from the U.S.). HINT1 expression in tissues was detected by immunohistochemistry. The frequencies of hypermethylation of *Hint1* in tumor, paired adjacent non-tumor and normal liver tissue were 55.0%, 37.5% and 9.1%, respectively. A statistically significant inverse association was found between *Hint1* methylation status and expression of the HINT1 protein in tumor tissues ($p<0.003$). The relationship between *Hint1* methylation status and clinical features and other, previously measured biomarkers was also analyzed. *p16* hypermethylation was statistically significantly associated with *Hint1* methylation status ($p=0.035$). There were no correlations between *Hint1* methylation and HBV or HCV infection status or AFB_1- and PAH-DNA adduct levels. These results suggest that promoter hypermethylation of *Hint1* may play a role in hepatocarcinogenesis.

Keywords

Hint1; HCC; epigenetic changes; promoter hypermethylation; *p16*; environmental carcinogens

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Conflicts of Interest Statement
None Declared

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies in the world, and a leading cause of death in many countries. The epidemiology of HCC has marked demographic and geographic variations, occurring mainly in Africa and Asia. However, the incidence is also increasing in the United States and Europe (1). The major risks for the development of HCC have been identified as chronic hepatitis B virus (HBV) and hepatitis virus C (HCV) infections and several dietary or environmental factors, including aflatoxin B_1 (AFB_1) and polycyclic aromatic hydrocarbons (PAHs). HBV and HCV infections and AFB_1 exposure are responsible for approximately 80% of all HCCs (2;3). As with other cancers, the development of HCC is a complex, multistep process (4). The molecular pathogenesis of HCC appears to involve multiple genetic aberrations in the molecular control of hepatocyte proliferation, differentiation and death and the maintenance of genomic integrity. This process is influenced by the cumulative activation and inactivation of oncogenes, tumor suppressor genes, cell cycle control genes and other genes.

The HINT protein, a member of the histidine triad (HIT) family, is highly conserved in diverse species and ubiquitously expressed in mammalian tissues. The HIT protein superfamily consists of at least three subfamilies: Hint, Fhit and Ga1T (5). In previous studies, *Hint1* deleted mice had a marked increase in susceptibility to chemical carcinogen-induced gastric tumors (6), mammary tumors and ovarian tumors (7). In addition, with aging, *Hint1* deleted mice displayed an increase in the occurrence of a variety of spontaneous tumors including HCC (7). These studies in mice also provided evidence that *Hint1* may be haplosufficient with respect to its function as a tumor suppressor gene. Mechanistic studies indicate that *Hint1* can play a role in apoptosis and *p53* expression (8) and that it can bind to and inhibit several transcription factors including MITF, USF2 and β-catenin and also inhibit AP1 activity by binding to POSH (9). It had been reported that *Hint1* is transcriptionally silenced in some human non-small cell lung cancer (NSCLC) cell lines and that increased expression of *Hint1* inhibits growth of the NSCLC cell lines H522 and H538 (10). Similar effects have been seen in colon cancer cells (9).

During the past decade, extensive studies in the field of epigenetics have brought an awareness that not only genetic, but also epigenetic changes, play a very important role in carcinogenesis (11;12). DNA methylation is one of the best understood epigenetic mechanisms; hypermethylation of normally unmethylated CpG islands, which are CpG dinucleotide-rich areas located mainly in the promoter regions of many genes, correlate with loss of transcription and loss of gene function (13). In HCC, a growing number of genes have been identified as undergoing aberrant promoter hypermethylation, suggesting that promoter hypermethylation is an important molecular mechanism for hepatocarcinogenesis. These include the genes *p16, p15* and *RASSF1A* (14;15). These epigenetic changes have also been implicated as early events in the development of HCC (16–18). In recent studies, we found promoter hypermethylation of *Hint1* in a subset of both colon cancer and HCC cell lines (9;19).

With the above findings as a background, in the present study, we investigated the *Hint1* promoter methylation profile in HCC and paired adjacent non-tumor DNA samples from patients and also explored the correlation between *Hint1* methylation status and other biomarkers and clinical parameters.

Materials and methods

Patient population and data on clinical parameters

The study samples consisted of 40 frozen dissected tumor and paired adjacent non-tumor tissues, collected in the Department of Surgery, National Taiwan University Hospital. Informed consent was obtained from patients, and the study was approved by the appropriate institutional review committees. Demographic data and clinicopathological characteristics were obtained from hospital charts, and HBV and HCV status was determined by immunoassay (see Table 1.). Fourteen normal control liver tissues were obtained from subjects affected with intrahepatic stones, liver cysts, and other non-cancerous diseases identified at the National Taiwan University Hospital. Eight U.S. normal control liver tissues were from subjects affected with heart disease identified at Columbia Presbyterian Hospital in New York City.

Immunohistochemical detection of Hint1 protein in paraffin-embedded sections

Detection of the HINT1 protein in 5 μm paraffin-embedded sections used a commercial polyclonal antibody (ProteinTech Group Inc. Campbell Park Dr., Chicago, IL). After deparaffinization and rehydration in graded ethanol, the slides were immersed in 10 mM citric acid (pH 6.0) and microwaved for 10 min at 400 W. Staining was carried out according to the manufacture's instruction: the primary antibody (1:100 dilution) was added and sections were incubated overnight at 4°C. This was followed by adding the secondary antibody and ABC reagent and DAB (both ABC and DAB kits were from Vector Laboratories, Burlingame, CA). Slides were then counterstained with Harris hematoxylin (Sigma, St. Louis, MO). The following categories were used for scoring: intensity of staining, none (0), mild (1), moderate (2), strong (3); and percentage of positive staining, <5% (0), 5–25% (1), 25–50% (2), >50% (3) of cells (20). Combining intensity and percentage staining resulted in the following score 0–1; negative (−); 2–6 positive (+). Liver sections from wild type (*Hint1*+/+) and *Hint1* knocked out (*Hint1*−/−) mice (7) were used as positive and negative controls.

DNA extraction

DNA was isolated from frozen tissue samples, as previous described (21). Briefly, tissue was placed in liquid nitrogen and pulverized with a blender. The tissue powder was lysed with a DNA lysing buffer (10 mM Tris, 10 mM NaCl, 0.1% sodium dodecyl sulfate at pH 7.9, and 200μg/ml proteinase K). DNA was isolated by RNase treatment, phenol/chloroform extraction and ethanol precipitation.

Analysis of Hint1 and p16 hypermethylation status: methylation–specific polymerase chain reaction (MSP)

MSP was carried out, essentially, as described previously (22) and was based on the principle that treating DNA with sodium bisulfite results in the conversion of unmethylated citosine residues into uracil. Thus, the sequence of the treated DNA will differ if the DNA is originally methylated, and is then distinguishable by sequence-specific PCR primers. Bisulfite modification of tissue DNA was conducted with the CpGnome DNA modification kit (Chemicon International, Temecula, CA). The sense and antisense primers for the methylated *Hint1* promoter were 5′-TTTGCGTAGGTTTGGTTGC-3′ and 5′-AACAATCTCATCTACCATCTCGAC-3′, respectively, and the primers to detect the unmethylated *Hint1* were 5′-TATTTGTGAGGTTTGGTTGTGT-3′ and 5′-AACAATCTCATCTACCATCTCAAC-3′, respectively. PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining. Universal methylated DNA

(Chemicon International) was used as a positive control with distilled water as a negative control.

Data on *p16* methylation and AFB_1- and PAH-DNA adducts on these samples were available from our previous studies (14).

Statistical analysis

Fisher's exact tests were performed to evaluate the significance of the differences between the frequencies of *Hint1* promoter hypermethylation status of the various tissue categories, comparisons with Hint1 protein expression status, and comparisons with clinical characteristics.

Results

Hint1 methylation status

Methylation of the promoter region of *Hint1*, determined by MSP, was frequent in the HCC tumors, with 22 of 40 (55.0%) samples positive. For the paired adjacent non-tumor tissue samples, 15 of 40 samples were positive samples (37.5%) (Table 2). Representative examples of the gel analysis of MSP are shown in Figure 1. Interestingly, unmethylated *Hint1* alleles were also detected in all of samples. In the 22 normal liver controls, 14 samples were from Taiwan; 2 of 22 had promoter hypermethylation, none of the U.S. normal controls liver samples had promoter hypermethylation.

Expression of the Hint1 protein in HCC tissue samples

To determine whether the hypermethylation of CpG islands in the promoter region of *Hint1* in HCC tissues was correlated with loss of expression of the HINT1 protein, immunohistochemical staining using an anti-HINT1 antibody was carried out on 40 HCC tissue sections. Representative examples of HINT1 protein expression are shown in Figure 2(A and B). Fifteen of the 18 unmethylated HCC samples (83%) demonstrated positive nuclear and cytoplasmic staining and 14 of 22 methylated HCCs (64%) showed loss of expression of Hint1 (Table 2). Thus, the immunostaining results were strongly correlated ($p<0.003$) with *Hint1* methylation status (Table 3).

Relationship between Hint1 hypermethylation and clinical parameters and other biomarkers

Possible associations between the methylation status of *Hint1* and tumor stage, liver cirrhosis status, HBV infection status, *p16* methylation status and levels of AFB_1- and PAH-DNA adducts were investigated (Table 3). We found that *p16* hypermethylation was statistically significantly associated with *Hint1* methylation status ($p=0.035$), but there was no significant correlation with the other parameters.

Discussion

Hypermethylation of CpG islands in their promoter regions is an important mechanism for loss of function of several tumor suppressor genes, DNA repair genes and other genes in various types of human cancer (13). An increasing number of genes have been reported to undergo CpG island hypermethylation in HCC, which indicates the potential role of epigenetics in hepatocarcinogenesis (23). The promoters of ras association domain family 1A (*RASSF1A*) (14), $p16^{INK4a}$ (24), $p15^{INK4b}$ (25), O^6-methylguanine-DNA methyltransferase (*MGMT)* (26), glutathione *S*-transferase pi (*GSTP1*) (16;27), suppressor of cytokine signaling 1 (*SOCS-1*) (28), adenomatous polyposis coli (*APC*) (16) and E-cadherin (*E-Cad*) (29) are the most frequently methylated in HCC. These findings suggest

that CpG island hypermethylation is an important molecular mechanism in the development of HCC.

In previous investigations with genetically engineered mice, evidence was obtained that *Hint1* is a novel hapoinsufficient tumor suppressor gene (7). However, its precise mechanism of action and relevance to specific types of human cancer is still not clear. We found a low level of expression of the HINT1 protein in the SW480 cell line when compared with four other human colon cancer cell lines and obtained evidence that this is due to methylation of the promoter region of *Hint1* (9). In recent studies, we also found a low level of expression of the HINT1 protein in the human HCC cell lines HepG2 and Hep3B when compared with the human HCC cell line Huh7, by western blot analysis, and that this was also due to promoter methylation in the HepG2 and Hep3B cell lines (19). Other investigators found decreased expression of *Hint1* in a subset of human NSCLC cell lines, which appeared to be due to promoter hypermethylation based on studies utilizing 5-Aza-dC (10). Thus, decreased expression of HINT1 due to hypermethylation of the promoter region of the *Hint1* gene can occur in at least 3 types of human cancer cell lines.

Based on the above findings, in the present study, we investigated promoter hypermethylation of *Hint1* in DNA samples from primary HCC, paired adjacent non-tumor tissues from patients with HCC, and normal liver tissue DNA. Twenty two of the 40 (55.0%) HCC samples displayed *Hint1* gene promoter hypermethylation. Methylation was also observed in 37.5% of the paired adjacent nontumor tissues. This may be due to the fact that these tissue samples were not microdissected and, therefore, they may have been contaminated with a small population of HCC cells. Alternatively, since most of the adjacent nontumorous tissues are cirrhotic, promoter hypermethylation of *Hint1* may be an early event in hepatocarcinogenesis (17), as is the case with other tumor suppressor genes (30). Interestingly, hypermethylation of *Hint1* was also found in two normal control liver tissues, both from Taiwan and both HBV positive, but not in the 8 normal control liver tissues from the U.S. which were HBV negative. In accordance with our findings, DNA methylation of other tumor suppressor gene has been detected at a low frequency in histologically normal liver tissues (31).

In the present study, the HINT1 protein was detected in 15 of 18 (83%) tumor tissues with *Hint1* promoter not methylated and 8 of 22 (36%) tumor tissues with *Hint1* promoter methylated (p value for Fisher's exact test=0.003), suggesting that methylation status correlates inversely with HINT1 expression. The discordant data may be due to the lack of tissue microdissection resulting in contamination of the tumor tissue with adjacent nontumor tissue. Immunohistochemical staining of small pieces of tissue also limits the detection of protein expression and this may also help explain the discordant data. Discrepancies between Methylation Specific PCR (MSP) and immunohistochemistry detection were reported previously (26;32).

Promoter hypermethylation of some genes is significantly linked to pathological or clinical parameters. For example, *p16* hypermethylation is associated with HBV infection and expression of the HBV × protein (33;34). In our previous studies, statistically significant associations were found between *RASSF1A, p16,* and *MGMT* methylation status and the levels of AFB_1-DNA adducts in Taiwan HCC samples (14;26). *SOCS-1* silencing is significantly involved in the development of HCC from liver cirrhosis (28), and hypermethylation of *E-Cad* or *GSTP1* correlates with poor survival in HCC patients (23). In the present study, the correlations between *Hint1* methylation and HBV and HCV infections status and AFB_1- and PAH-DNA adduct levels were also investigated, but no statistically significant correlations were found. Perhaps, HBV and HCV infections and chemical

carcinogens like AFB_1 and PAHs do not affect *Hint1* promoter methylation status, although the relatively small sample size may limit this analysis.

Previous studies determined the frequency and chronology of methylation events of specific genes during the multistep process of hepatocarcinogenesis from cirrhosis to HCC. CpG island hypermethylation occurs in the premalignant stages and tends to accumulate during multistep hepatocarcinogenesis. The data suggest that CpG island hypermethylation of *COX-2* or *p16* might be potential molecular markers for the identification of patients with chronic liver disease at high risk for progression to HCC (23). Another study suggested that the most striking methylation pattern in HCC is the concurrent methylation of multiple tumor suppressor genes (TSG). Although methylation of one or two genes can be observed in nontumor and cirrhotic liver tissue, the majority of HCC cases harbored three or more methylated TSGs (16). In present study, *Hint1* methylation was significantly associated with *p16* hypermethylation (p=0.035). This finding is consistent with previous studies and provides further evidence that several TSGs genes accumulate methylation during progression from pre-malignant stages to HCC.

Our previous mechanistic studies suggested that Hint1 inhibits AP-1 activity by binding to a POSH-JNK2 complex, thus inhibiting the phosphorylation of c-Jun; this effect could contribute to the tumor suppressor activity of Hint1 (9). Decreased expression of the *Hint1* gene through epigenetic silencing may play a role in enhancing the growth of a subset of human hepatoma cell lines by increasing the expression of genes controlled by the transcription factors β-catenin, USF2 and NFB (19).

In conclusion, to the best of our knowledge, this is the first study on detection of *Hint1* promoter hypermethylation in tumor tissues. Our investigation demonstrated that epigenetic inactivation of *Hint1* is a frequent event in the development of HCC and promoter hypermethylation of *Hint1* can be an early event in hepatocarcinogenesis. The biologic basis and mechanisms of *Hint1* inactivation by hypermethylation and the relationship between epigenetic changes in *Hint1* and other risk factors for HCC is not clear at the present time. Additional studies with larger sample sizes are required to elucidate these aspects. We believe that these studies of environment-epigenetic interactions are necessary for a deeper understanding of HCC and the mechanism of action of carcinogenic exposures and could be used to identify novel opportunities for the prevention and therapy of HCC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abreviation used are

Hint1	Hit nucleotide binding protein-1
HCC	hepatocellular carcinoma
HBV	hepatitis B virus
HCV	hepatitis C virus

AFB_1	aflatoxin B_1
PAHs	polycyclic aromatic hydrocarbons

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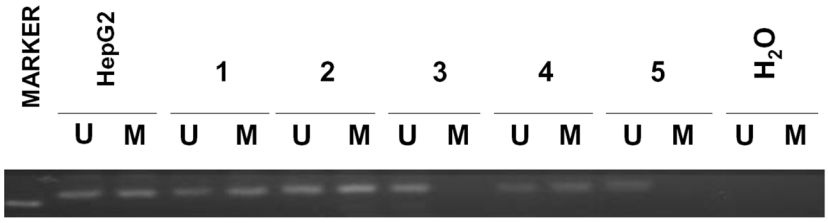

Figure 1.
Methylation analysis of *Hint1* in DNA from HCC tissues, the HepG2 HCC cell line was used as a positive control. l. Bisulfite-treated DNA was used for PCR amplification using primers sets designed for methylated (m) and unmethylated (u) *Hint1.* M, molecular weight marker (100 bp); 1, DNA from the HepG2 HCC cell line; lines 2–6 DNA from 5 HCC cases; line 7, distilled water as negative control.

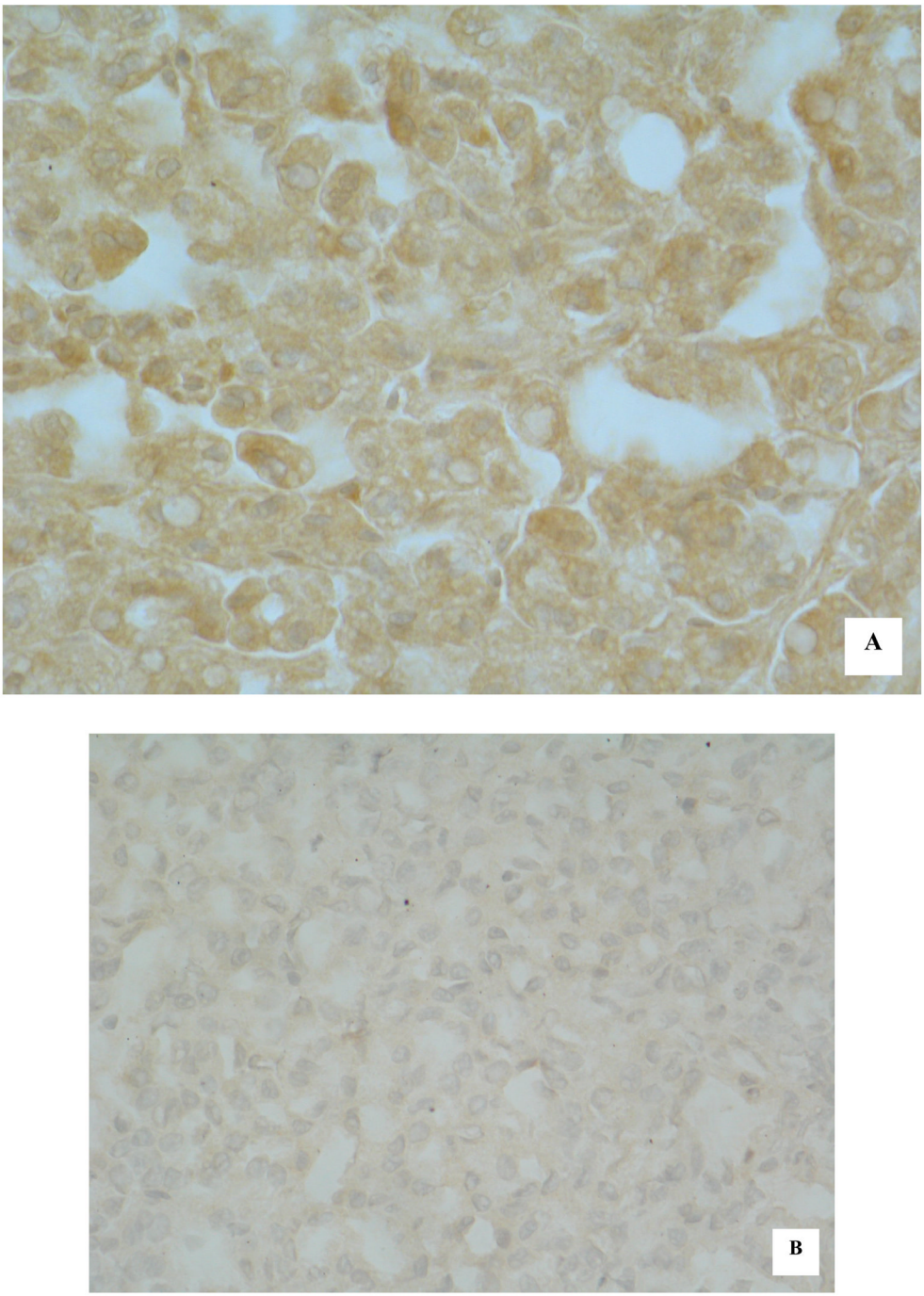

Figure 2.
Immunohistochemical detection of HINT1 expression in HCC samples. (A) HCC tissue with unmethylated *Hint1* illustrating expression of the protein in both tumor cell nuclei and cytoplasm. X400; (B) HCC tissue with methylated *Hint1* illustrating lack of expression in tumor cells. X400

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Table 1

Demographics, HCV status, HBsAg status and tumor characteristics of HCC cases

ID	Age	Gender[1]	Cirrhosis in nontumorous tissue	Grade	Anti-HCV	HBsAg
F003	NA[2]	NA	NA	NA	NA	NA
F004	67	M	+	II	+	+
F005	47	M	−	II	−	+
F006	70	F	−	I	+	−
F007	NA	NA	NA	NA	NA	NA
F008	NA	M	NA	NA	NA	NA
F009	56	M	−	NA	NA	+
F011	NA	NA	NA	NA	NA	NA
F012	39	M	−	III	+	+
F013	78	M	+	II–III	−	+
F014	42	M	−	III	−	+
F015	NA	NA	NA	NA	NA	NA
F017	70	F	−	II	+	−
F018	51	M	−	II	−	+
F019	20	M	−	I–II	−	+
F020	NA	NA	NA	NA	NA	NA
F021	45	M	−	II	−	+
F022	58	M	+	I	+	−
F023	43	M	−	II	−	+
F025	51	M	−	II	+	−
F028	35	F	−	III	NA	+
F029	49	M	+	II	−	+
F030	37	M	−	II	−	+
F032	50	M	−	II	−	+
F033	48	M	−	III	+	+
F034	44	M	−	I–II	NA	+
F035	49	M	+	III–IV	−	+
F036	62	F	+	III	+	+

ID	Age	Gender[1]	Cirrhosis in nontumorous tissue	Grade	Anti-HCV	HBsAg
F038	62	M	+	II	+	+
F039	69	M	+	III	−	−
F040	76	M	−	III	−	+
F042	40	M	−	II	NA	+
F043	59	F	+	III	−	+
F044	NA	NA	NA	NA	NA	NA
F045	63	M	+	II	−	+
F047	45	M	+	II	−	+
F048	68	M	+	II	NA	+
B115	45	M	+	NA	−	+
B169	53	M	NA	NA	−	−
D145	58	M	NA	NA	+	NA

[1]M, male; F, female;

[2]NA, data not avalable

Table 2

Hint1 Gene promoter hypermethylation status and protein expression in Taiwan HCC samples

	Hypermethylation status		Protein expression
	Tumor	Adjacent non-tumor	Tumor
3	−	−	+
4	−	−	+
5	−	−	+
6	−	+	+
7	+	+	−
8	−	−	+
9	−	−	+
11	+	−	+
12	+	−	−
13	+	+	−
14	−	−	+
15	+		−
17	−	+	−
18	−	+	−
19	−	−	+
20	+	−	−
21	−	−	+
22	+	+	−
23	+	+	−
25	+	+	−
28	+	+	+
29	−	−	−
30	+	+	+
32	+	+	+
33	+	−	−
34	+	+	−
35	+	+	+
36	+	+	+
38	+	−	+
39	−	−	+
40	+	−	+
42	−	−	+
43	+	−	−
44	−	−	+
45	−	−	+
47	+	−	−
48	−	−	+
B115	−	−	+

	Hypermethylation status		Protein expression
	Tumor	Adjacent non-tumor	Tumor
B169	+	−	−
D145	+	+	−

Table 3

The association of clinical characteristics with *Hint1* methylation status

	Tumor tissues		
Variable	**Non-methylated**	**Methylated**	***P***
Adjacent non-tumor tissues			
Non-methylated	15	9	0.01
Methylated	3	12	
Missing	0	1	
Hint 1 protein expression			
No	3	14	0.003
Yes	15	8	
Grade			
I orII	11	8	0.49
IIIor IV	2	8	
Missing	5	6	
Liver cirrhosis			
No	9	9	0.71
Yes	6	7	
Missing	3	6	
HBsAg			
Negative	3	2	0.44
Positive	12	15	
Missing	3	5	
Aflatoxin B1-adducts			
Low	11	7	0.07
Medium	3	11	
High	4	4	
PAH-adducts			
Low	9	13	0.49
Medium	4	7	
High	4	2	
Missing	1	0	
p16 methylation			
No	10	5	0.035
Yes	8	17	