Hypomethylated P4 Promoter Induces Expression of the *Insulin-Like Growth Factor-II* Gene in Hepatocellular Carcinoma in a Chinese Population

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Abstract

Purpose: The expression of human *insulin-like growth factor-II* (*IGF-II*) is regulated by the activation of four promoters (P1-P4) acting in a development-dependent, tissue-specific manner. *IGF-II* overexpression associated with P3 and P4 activation is observed in animal and human hepatocarcinogenesis. We correlated P4 epigenetic alteration with P4 transcript activation and clinicopathologic features.

Experimental Design: We analyzed P4 epigenetic alteration using methylation-specific PCR in 34 hepatocellular carcinoma (HCC) specimens, 34 matched adjacent nontumor specimens, and 8 normal adult liver specimens. The data were correlated with activation of P4 transcription by using reverse transcription-PCR. Epigenetic alteration was compared with patients' clinicopathologic features.

Results: Compared with normal liver tissue, hypomethylation of P4 CpG islands was significantly more frequent in HCC ($P = 0.03$) and matched tissues ($P = 0.047$). P4 mRNA levels in HCC with unmethylated alleles were significantly higher than in HCC without unmethylated alleles ($P = 0.001$); P4 mRNA levels in matched nontumor tissues with unmethylated alleles were significantly higher than in matched nontumor tissues without unmethylated alleles ($P = 0.005$). P4 hypomethylation in HCC was associated with portal vein tumor embolus ($P = 0.017$) and poorer tumor differentiation ($P = 0.025$).

Conclusions: These findings suggest that *IGF-II* P4 hypomethylation may be an early and frequent event and that it may contribute to P4 transcription expression activation during the transformation of a premalignant liver lesion to HCC. Furthermore, aberrant hypomethylation of P4 CpG islands not only may play an important role during hepatocarcinogenesis but might also be a useful biomarker for poor prognosis of patients with HCC.

Methylation of DNA, an epigenetic modification, plays important roles in silencing X-linked and imprinted genes, chromatin remodeling, and genome stability (1–4). Aberrant methylation has been shown to occur in human carcinogenesis (3–6). Genome-wide hypomethylation (7–12) and regional hypermethylation of the promoter regions of critical tumor suppressor genes (13–15) have been observed in several human cancers, including hepatocellular carcinoma (HCC). The former may lead to activation of genes, such as proto-oncogenes, and reexpression of provirus sequences; the latter may result in silencing the expression of tumor suppressor genes (16–20). To date, however, there have been no studies that have shown how specific promoter region methylation affects gene expression in HCC.

Evidence shows that activation of the human *insulin-like growth factor II* (*IGF-II*) signaling pathway is involved in hepatocarcinogenesis. *IGF-II* is an autocrine and paracrine growth factor and a cell mitogen that exerts its bioactivity through interaction with the *IGF-I* receptor. The *IGF-II* peptide is inactivated by the mannose-6-phosphate receptor/*IGF-II* receptor, which binds *IGF-II* on the cell surface and transports it to lysosomes, where degradation occurs (21, 22). The gene that encodes *IGF-II* consists of nine exons and is transcribed from four different promoters (P1-P4). Multiple transcripts are synthesized as a result of alternate promoter usage and the splicing of the unique 5′-untranslated region to common coding exons (23–25). The *IGF-II* gene is transcriptionally regulated in a development-dependent and tissue-specific manner. In the fetal liver, the promoters P2, P3, and P4 are active; P3 is the most active promoter, and P1 is inactive. However, in the adult liver, P1 becomes

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dominant and produces ~50% of the total *IGF-II* transcripts, and P2 to P4 activities are decreased or lost (26, 27).

An elevated expression of *IGF-II* has been found in both liver tumors and preneoplastic hepatic foci in different animal hepatocarcinogenesis models (28–30) as well as in human HCC (31–33). *IGF-II* overexpression in HCC is associated with significant up-regulation of the fetal transcripts driven by P3 and P4 promoters (34). However, the mechanisms responsible for up-regulation of fetal transcripts due to P3 and P4 activity during hepatocarcinogenesis are not well understood.

Generally, alterations in the level of DNA methylation of CpG islands located around promoter regions of eukaryotic genes promote or suppress expression of these genes (3, 4, 10–12). Because the methylation status of the P4 promoter for *IGF-II* has not yet been studied in HCC, the present study was done to determine whether DNA hypomethylation occurs in the P4 promoter region and, if so, to examine the nature of its relationship to P4-driven *IGF-II* mRNA expression. Our objective was to obtain a better understanding of transcriptional regulation mechanisms by which P4 promoter activity is controlled during human hepatocarcinogenesis as well as to determine if there are any correlations between P4 promoter methylation status and clinicopathologic variables.

Materials and Methods

Tissue specimens. Tumor tissue specimens and matched tissue specimens consisting of adjacent nontumor tissue (taken within 2 cm of the tumor margin) were obtained during surgery from 34 consecutive HCC patients (28 men and 6 women) treated at the First Affiliated Hospital of Jinan University (Guangzhou, China). Controls consisted of normal adult liver tissue samples obtained from 8 adults (7 men and 1 woman; mean age, 44.4 years; range, 35-52 years). The only source of control samples was the relatively few patients who suffered accidental

Table 1. Clinical and pathologic characteristics of patients with HCC

Case	Gender	Age (y)	Tumor size (cm)	HMNL	TEPV	α-Fetoprotein (μg/L)	HCI	TMF	Diagnosis (Edmonson class)
2	M	57	4.5	Cirrhosis	+	1,056	–	–	HCC (IV)
5	M	74	7.5	Cirrhosis	+	7,369	+	+	HCC (IV)
8	M	39	9	Chronic hepatitis	+	3,856	+	+	HCC (IV)
11	M	44	10	Cirrhosis	+	542	+	+	HCC (IV)
18	M	67	12.5	Cirrhosis	+	1,075	–	+	HCC (IV)
19	F	44	3.5	Cirrhosis	+	3,703	+	–	HCC (IV)
23	M	47	13	Cirrhosis	+	3,570	+	–	HCC (IV)
24	M	57	11	Chronic hepatitis	+	458	+	+	HCC (IV)
6	M	40	4	Cirrhosis	–	385	–	–	HCC (IV)
28	F	68	6.5	Cirrhosis	–	742	–	+	HCC (IV)
3	F	40	10	Cirrhosis	+	1,537	+	+	HCC (III)
9	M	48	11	Cirrhosis	+	14	–	+	HCC (III)
14	M	42	3.5	Cirrhosis	+	436	+	+	HCC (III)
15	M	45	8.5	Cirrhosis	+	2,120	+	–	HCC (III)
20	M	38	2.5	Cirrhosis	+	59	+	–	HCC (III)
22	M	63	9.5	Cirrhosis	+	11,560	–	+	HCC (III)
30	M	46	2.5	Cirrhosis	+	978	+	+	HCC (III)
32	M	48	12.5	Chronic hepatitis	+	9	+	+	HCC (III)
34	M	58	7.5	Chronic hepatitis	+	42,795	–	–	HCC (III)
27	M	65	4.7	Chronic hepatitis	–	3,421	+	–	HCC (III)
1	M	42	2.5	Cirrhosis	–	49	–	–	HCC (II)
4	M	39	5.5	Chronic hepatitis	–	150	+	–	HCC (II)
10	M	41	2.5	Cirrhosis	–	3	–	–	HCC (II)
12	M	60	5.5	Chronic hepatitis	–	10	–	–	HCC (II)
13	F	50	6.5	Cirrhosis	–	8,845	–	+	HCC (II)
16	M	41	5.5	Cirrhosis	–	369	+	+	HCC (II)
26	M	62	17.5	Cirrhosis	–	125	+	–	HCC (II)
31	M	37	4	Normal	–	12,389	–	–	HCC (II)
25	F	48	12.5	Cirrhosis	+	12	–	–	HCC (I)
29	M	49	8.8	Cirrhosis	+	3,267	–	–	HCC (I)
7	M	44	5	Cirrhosis	–	7	+	–	HCC (I)
17	M	70	11.5	Normal	–	85	–	+	HCC (I)
21	M	61	7.5	Chronic hepatitis	–	578	+	–	HCC (I)
33	F	53	4.6	Cirrhosis	–	6,376	–	+	HCC (I)

Abbreviations: HMNL, histology of matched nontumor liver; TEPV, tumor embolus of portal vein; HCI, hepatocapsular invasion; TMF, tumor microsatellite formation.

liver injury (3 cases of hepatic rupture) or had liver surgery (5 cases of hepatic hemangioma). Because gender has not been found to be a relevant correlate in HCC, data for men and women were combined. All tissue samples were snap frozen and kept at −80°C until use. Study approval was secured from the medical ethics committee of the First Affiliated Hospital of Jinan University, and informed consent was obtained from all subjects before tissue sample collection.

Histologic examination. The presence of chronic hepatitis, cirrhosis, or both in matched adjacent nontumor tissue samples was evaluated based on the criteria of Desmet et al. (35). The degree of differentiation of the HCC tumor samples was determined according to the criteria of Edmondson and Steiner (36).

DNA extraction. Total genomic DNA was extracted from the frozen liver tissue specimens using the QIAamp Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Sodium bisulfite modification and methylation-specific PCR. Sodium bisulfite modification and methylation-specific PCR (MSP) were conducted based on the principle that the bisulfite treatment of genomic DNA can convert unmethylated cytosine residues into uracil, whereas methylated cytosine residues remain unmodified. Thus, after bisulfite conversion, methylated and unmethylated DNA sequences can be distinguished using sequence-specific PCR primers (37, 38). Bisulfite modification of 1 μg DNA was carried out using the CpGenome DNA Modification kit (Serologicals Corp., Norcross, GA) according to the manufacturer's protocols. The modified DNA was precipitated with ethanol and resuspended in TE buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA) and used immediately or stored at −80°C until use.

MSP was done to identify the methylation status of the P4 promoter CpG islands of *IGF-II* in HCC specimens, matched adjacent nontumor specimens, and normal adult liver specimens. The primer pairs for MSP were designed according to the complete sequence of *IGF-II* (NT_009308) using CpG Ware Primer Design Software (Serologicals). The sense and antisense primers for methylation-specific DNA (amplified fragment of 181 bp) were 5′-TTATATGTGTGATTCGTGT-TTTGC-3′ and 5′-CTACCATAACTCCTACCAACTCGAC-3′, respectively. The sense and antisense sequences of unmethylation-specific primers (amplified fragment of 180 bp) were 5′-TATATGTGTGATTTGTGTTTT-GTGG-3′ and 5′-CTACCATAACTCCTACCAACTCAAC-3′, respectively. PCR was conducted in a 25-μL reaction volume using *LA Taq* polymerase (TaKaRa, Kyoto, Japan). The PCR conditions were as follows: 1 cycle at 95°C for 10 minutes followed by 30 cycles of 94°C for 45 seconds, 59°C (methylation-MSP) or 57°C (unmethylation-MSP) for 45 seconds, 72°C for 45 seconds, and a final extension at 72°C for 5 minutes. PCR products were separated on 2% agarose gels and visualized under UV illumination after ethidium bromide staining.

RNA isolation. Total RNA was prepared from frozen tissue samples (10-20 mg) using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.

Reverse transcription-PCR. We synthesized cDNA using 10 units Reverse Transcriptase XL (avian myeloblastosis virus; TaKaRa) from 2 μg total RNA in a 20-μL volume containing 4 μL 5× avian myeloblastosis virus buffer, 2 μL 10 mmol/L deoxynucleotide triphosphates, 20 units RNase inhibitor, and 50 pmol oligo(dT)18 (TaKaRa). The reaction mixture was incubated at 25°C for 10 minutes and at 42°C for 60 minutes. The reaction was then terminated by incubation at 95°C for 10 minutes. To control contamination by genomic DNA, all RNA samples were run in duplicate with or without the addition of reverse transcriptase.

The primers for P4-specific *IGF-II* transcripts were 5′-GAGCCTT-CTGCTGAGCTGTAG-3′ and 5′-GTAGCACAGTACGTCTCCAG-3′ (293 bp; ref. 39). The glyceraldehyde-3-phosphate dehydrogenase–specific primers (internal control) were 5′-CACCATCTTCCAGGAGCGAG-3′ and 5′-TCACGCCACAGTTTCCCGGA-3′ (372 bp; ref. 39). P4-specific transcripts were coamplified with glyceraldehyde-3-phosphate dehydrogenase in the presence of 5 μL cDNA, 25 μL 2× GC buffer I, 4 μL of 2.5 mmol/L deoxynucleotide triphosphates, 50 pmol each forward and reverse primer pair, and 2.5 units *LA Taq* polymerase. After initial denaturation at 94°C for 3 minutes, PCR was carried out using the following program: 94°C, 57°C, and 72°C for 1 minute for 30 cycles followed by a final extension at 72°C for 10 minutes.

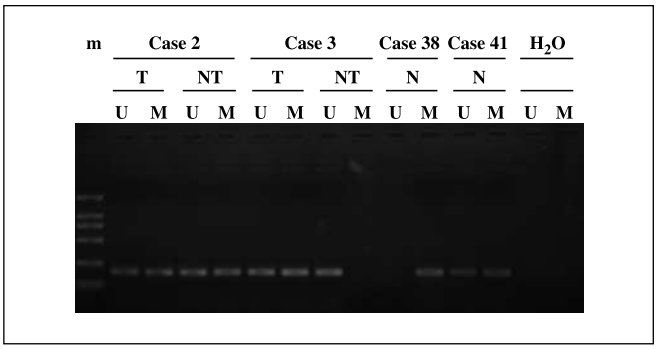

Fig. 1. Representative MSP analysis results of P4 promoter methylation status of the *IGF-II* gene in HCC tissue samples, matched adjacent nontumor tissue samples, and normal adult liver tissue samples. Lane U, the presence of a 180-bp PCR product indicates the existence of an unmethylated allele in the P4 promoter; Lane M, the presence of a 181-bp product represents the presence of a methylated allele in the P4 promoter. Water served as the negative control. *T,* tumor tissue; *NT,* matched nontumor tissue; *N,* normal liver tissue; *m,* DL2000 marker.

PCR products (5 μL) were run on 2% agarose gels and quantified by densitometric scanning using a gel imaging system (MultiGenius, Syngene, Frederick, MD).

Statistical analysis. Categorical data were analyzed by χ^2 or Fisher's exact tests, depending on the absolute numbers included in the analysis, and the quantitative data were evaluated by ANOVA or the independent sample *t* test followed by Wilcoxon's rank-sum test using SPSS 12.0 software (SPSS, Inc., Chicago, IL). Results were considered statistically significant at $P < 0.05$.

Results

The clinical and pathologic characteristics of the HCC study population are presented in Table 1. The average age was 50.8 years (range, 37-74 years). Sixty-eight percent of the HCC patients had a tumor size ≥5 cm, and >50% had a tumor embolus of the portal vein. A majority of patients had an elevated α-fetoprotein level. Nearly 60% of the HCC patients' histology was classified as poorly differentiated (Edmonson class III-IV).

DNA methylation status of* IGF-II *P4 promoter in HCC. Figure 1 shows the MSP analysis results of the P4 promoter methylation status of the *IGF-II* gene in HCC tissue samples, matched adjacent nontumor tissue samples, and normal adult liver tissue samples. An unmethylated allele was detected in 25 of 34 (73.5%) HCC samples and in 24 of 34 (70.6%) matched nontumor samples, whereas the unmethylated allele was found in only 2 of 8 (25.0%) normal adult liver samples (Table 2). Of the 25 HCC and 24 matched tissue samples with an unmethylated allele, 15 HCC samples and 16 matched tissue samples were found to have only the unmethylated allele, whereas the remaining 10 HCC and 8 matched tissue samples had both methylated and unmethylated alleles. The 2 normal liver samples with an unmethylated allele also had the methylated allele. Hypomethylation of P4 promoter CpG islands was more frequent in HCC ($P = 0.03$) and matched tissue samples ($P = 0.047$) than in normal adult liver tissue samples.

P4 promoter hypomethylation and expression activation of* IGF-II *in HCC. To determine whether P4 promoter hypomethylation in HCC might be correlated with *IGF-II* expression

Table 2. Methylation status on *IGF-II* P4 mRNA expression

Case	Gender	Age (y)	P4 mRNA Levels					
			HCC tumor		HCC nontumor		Normal tissue	
			Unmethylated allele	Methylated allele	Unmethylated allele	Methylated allele	Unmethylated allele	Methylated allele
2	M	57	0.97		1.24			
5	M	74	0.75		1.69			
8	M	39	0.68		1.14			
11	M	44	1.21		1.37			
18	M	67	3.32		1.97			
19	F	44	1.39		1.83			
23	M	47		0.78		1.12		
24	M	57	0.95		0.61			
6	M	40	0.34			0.78		
28	F	68		0.44		0.48		
3	F	40	1.35		2.72			
9	M	48	0.73		1.95			
14	M	42	0.65		0.91			
15	M	45	0.56		0.48			
20	M	38	1.39		1.87			
22	M	63	2.89		1.97			
30	M	46	1.25		1.42			
32	M	48	1.86		2.12			
34	M	58	1.27		0.83			
27	M	65	1.33		1.23			
1	M	42	0.58		1.35			
4	M	39	0.61			0.67		
10	M	41	0.76		2.89			
12	M	60		0.00		0.96		
13	F	50		0.00	2.23			
16	M	41	2.47		1.79			
26	M	62	0.86		0.53			
31	M	37		0.47		0.32		
25	F	48		0.28		0.35		
29	M	49	0.75			0.00		
7	M	44		0.00	1.18			
17	M	70		0.00		0.62		
21	M	61		0.64		0.68		
33	F	53	0.73		0.46			
35	M	40						0.32
36	M	45						0.12
37	F	44						0
38	M	50						0
39	M	35						0.19
40	M	52						0.42
41	M	38						0.28
42	M	51						0.56

activation, semiquantitative reverse transcription-PCR was used to evaluate the expression levels of P4-driven *IGF-II* transcripts in all tissue samples. Representative results are shown in Fig. 2. P4 promoter expression was markedly up-regulated in most of the HCC and matched nontumor tissue samples compared with expression in normal tissue samples. P4 transcript levels in both HCC and matched nontumor tissue samples were significantly higher than in normal liver tissue samples (P = 0.037 and 0.002, respectively). No difference in P4 transcript levels was found between HCC and matched adjacent nontumor tissue samples (P = 0.324). P4 mRNA levels in both HCC and matched nontumor tissue samples with an unmethylated allele were significantly higher than in HCC and matched adjacent tissue mRNA without an unmethylated allele (P = 0.001 and P = 0.005, respectively; Table 3).

Correlation of P4 promoter hypomethylation with clinicopathologic characteristics of HCC. The unmethylated allele was more common in patients with portal vein tumor embolus

(P = 0.025) and in patients with poorly differentiated HCC tissue (P = 0.017). On the other hand, there was no association between the unmethylated allele frequency in P4 promoter CpG islands and age, α-fetoprotein level, tumor size, hepatocapsular invasion, or tumor microsatellite formation.

Discussion

In this preliminary study, we characterized the epigenetic changes found on the *IGF-II* P4 promoter in human HCC. The unmethylated allele was found in most of the HCC and matched nontumor tissue samples and it was more common in HCC patients, suggesting that P4 promoter hypomethylation may be an early and common event in the development of HCC. These results are consistent with those reported by Cho et al. (40), who found hypomethylation of the *CAGE* promoter at frequencies of >60% in breast, gastric, lung, and hepatic cancers. Furthermore, we detected both methylated and unmethylated alleles of the P4 promoter in HCC and adjacent nontumor tissue that could be due to heterozygous or partial demethylation of P4 promoter CpG islands among individual alleles or among precancerous liver and HCC cells.

Expression levels of P4 mRNA in the HCC and adjacent nontumor tissue samples were significantly higher, and P4 promoter hypomethylation was more common than in normal liver tissue samples. It is especially important to note that both HCC and adjacent nontumor tissues with the unmethylated allele showed a marked increase in P4 mRNA expression compared with both HCC and matched nontumorous tissues without the unmethylated allele, respectively. These findings suggest that P4 promoter hypomethylation may be closely related to up-regulation of P4 transcription and that hypomethylation-associated activation of P4 mRNA expression may play an important role in human hepatocarcinogenesis. Several studies that have examined hypomethylation of promoter regions in various cancers have reported similar results. Okada et al. (17) postulated that trefoil factor 3 promoter CpG hypomethylation may be one of the regulation mechanisms of trefoil factor 3 overexpression in HCC, and that it may be a critical process in mouse and human hepatocellular carcinogenesis. Xiao et al. (41) found that *MAGE-A1* mRNA expression in human hepatoma cell lines is associated with hypomethylation of the *MAGE-A1* promoter domain. Similarly, a recent report showed that the aberrant overexpression of cytochrome

Table 3. *IGF-II P4* mRNA expression levels by type of liver tissue

	No. cases	P4 mRNA level (mean ± SD)	*P*
Different liver samples			
HCC T	34	0.95 ± 0.76	0.037*, 0.324†
HCC NT	34	1.23 ± 0.72	0.002‡
N	8	0.24 ± 0.20	
HCC T			
Unmethylated allele	25	1.19 ± 0.74	0.001
Methylated allele	9	0.29 ± 0.31	
HCC NT			
Unmethylated allele	24	1.49 ± 0.67	0.000
Methylated allele	10	0.60 ± 0.33	

Abbreviations: T, tumor tissues; NT, matched nontumor tissues; N, normal liver tissues.
*HCC tumor tissues versus normal liver tissues.
†HCC tumor tissues versus HCC matched nontumor tissues.
‡HCC matched nontumor tissues versus normal liver tissues.

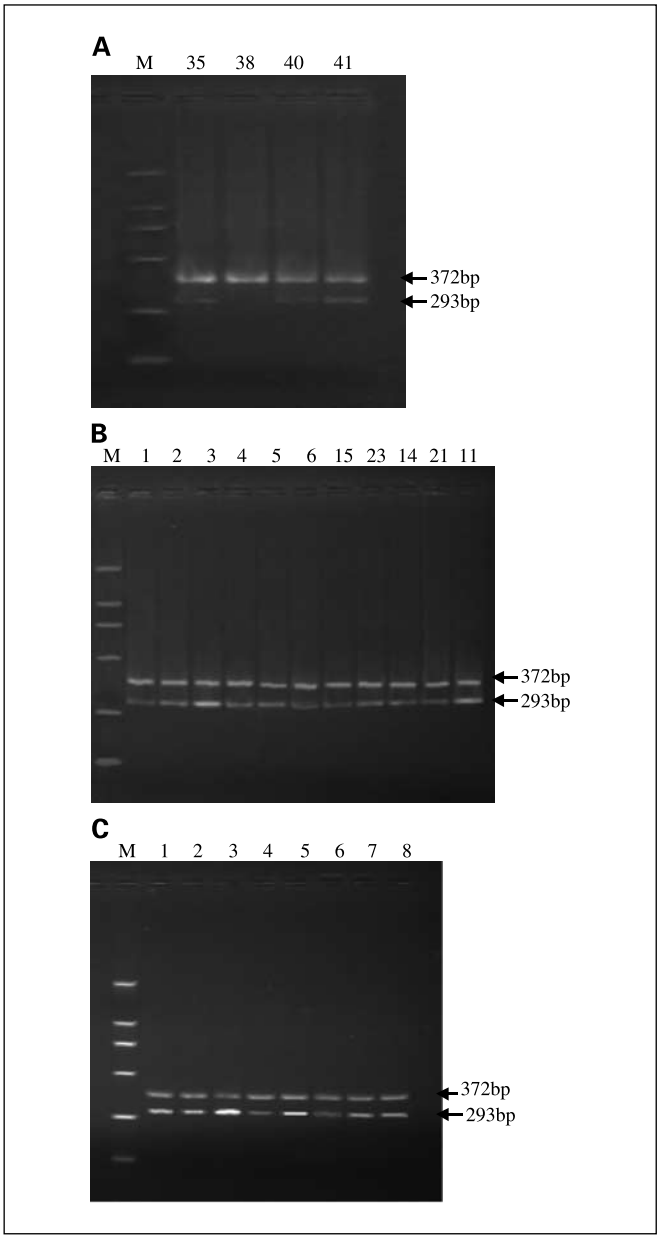

Fig. 2. Expression levels of *IGF-II* P4 transcripts in normal adult liver tissue (*A*) sample taken from patients with hepatic hemangioma or hepatic rupture, primary HCC (*B*), and matched adjacent nontumor liver tissue (*C*). Reverse transcription-PCR analysis of P4 promoter expression was done according to the description in Materials and Methods. The 293- and 372-bp fragments represent P4-specific transcripts and glyceraldehyde-3-phosphate dehydrogenase – specific transcripts, respectively. Top of each lane, patient's case number. *M,* DL2000 marker (TaKaRa).

P450 1B1 in prostate cancer may be regulated by hypomethylation of its gene promoter/enhancer region (42).

In our analysis of the associations between clinicopathologic characteristics and P4 promoter hypomethylation in HCC, the unmethylated allele was more frequent in HCC patients who had a tumor embolus in the portal vein and who had poorly differentiated tumors. Thus, it is possible that P4 promoter hypomethylation is positively associated with more malignant tumor behavior. If this were to be confirmed, then patients with higher P4 mRNA expression levels would have a poorer prognosis because tumor emboli in the portal vein and poor tumor differentiation are important prognostic factors in HCC patients (43).

In the high eukaryote genome, the covalent addition of a methyl group at the 5-carbon position of cytosine occurs specifically on the cytosines of CpG dinucleotides. Such DNA methylation plays a central role in controlling many genetic functions (44, 45). The enzymes involved in DNA methylation are responsible for the maintenance of the methylation status of the genomes after DNA replication (DNA methyltransferase I) and also act in *de novo* DNA methylation that occurs during the early development of high eukaryotic organisms (DNA methyltransferase IIIA and IIIB; ref. 3). There is accumulating evidence suggesting that aberrant DNA methylation (global hypomethylation and local hypermethylation) changes gene transcription and is implicated in tumorigenesis (3, 46, 47). Genome-wide hypomethylation, composed mainly of repetitive sequences (7–12), has been shown in several cancers, including HCC. Furthermore, hypomethylation is also observed in normally methylated CpG islands of promoter regions in human cancers (48, 49), where it appears to induce the aberrant expression of downstream genes. However, the mechanisms leading to hypomethylation of promoter regions, as well as when, how, and why hypomethylation occurs in cancers, are as yet unclear.

Numerous studies have found increased levels of *IGF-II* mRNA and/or protein in human HCC (32, 34, 50–52). The potential mechanisms for up-regulation of *IGF-II* include an allelic imbalance (53) and the activation of fetal P3 and P4 gene promoters (34). One mechanism that may be involved in the reexpression of fetal transcript in adult HCC is the hepatitis B virus X protein. Lee et al. (54) found that hepatitis B virus X protein increases endogenous *IGF-II* expression due to the fetal promoters P3 and P4. Analysis of the P4 promoter showed that the hepatitis B virus X gene product positively regulates *IGF-II* transcription by augmenting the DNA-binding activity of transcription factor Sp1. Furthermore, aflatoxin B1–induced mutation of *p53* at codon 249 (p53mt249) is crucial during HCC formation after hepatitis B virus infection. The p53mt249 mutation markedly increases *IGF-II* transcription, largely due to the P4 promoter, by enhancing the formation of transcriptional complexes via enhanced DNA-protein (Sp1 or TATA box–binding protein) and protein-protein (Sp1 and TATA box–binding protein) interactions (55). To date, experiments dealing with other possible mechanisms, such as changes in DNA methylation patterns that are involved in the activation of the P4 promoter, have not yet been done in HCC.

The small sample size of this preliminary analysis may have limited our ability to detect other significant associations, such as between some of the clinicopathologic characteristics (age, α-fetoprotein, tumor size, hepatocapsular invasion, and tumor microsatellite formation) and P4 promoter hypomethylation. The small sample size could have resulted in a selection bias that might have affected our findings as well. Given the encouraging results of our study, we are continuing to collect samples, and future reports will be based on a larger study population.

In conclusion, our preliminary data suggest that *IGF-II* P4 promoter hypomethylation may be an early and frequent event in hepatocarcinogenesis and that it may be involved in P4 transcription expression activation as the premalignant liver lesion progresses to HCC. This is the first study to show how the specific promoter region methylation affects gene expression in HCC. Furthermore, the abnormal hypomethylation of P4 CpG islands in patients with HCC may be associated with a poor prognosis and may thus serve as a useful molecular marker for predicting prognosis. Larger studies are needed to confirm these findings as well as to investigate the essentially unknown mechanisms of P4 hypomethylation.

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