DNA binding protein A expression and methylation status in hepatocellular carcinoma and the adjacent tissue

MAHMUT YASEN[1,4], GULANBAR OBULHASIM[3,4], KAZUNORI KAJINO[3], KAORU MOGUSHI[2], HIROSHI MIZUSHIMA[2], SHINJI TANAKA[1], HIROSHI TANAKA[2], OKIO HINO[3] and SHIGEKI ARII[1]

Departments of [1]Hepato-Biliary-Pancreatic Surgery and [2]Computational Biology and Bioinformatics, Tokyo Medical and Dental University, Tokyo 113-8510; [3]Department of Pathology and Oncology, Juntendo University School of Medicine, Tokyo 113-8421, Japan; [4]Department of Surgery, Xinjiang Uyghur Tumor Hospital of Xinjiang Medical University, Xinjiang 830011, P.R. China

Abstract. We investigated the expression and promoter methylation of dbpA in human hepatocellular carcinoma (HCC) and examined their correlation with clinicopathological features. In 96 paired samples of HCC and adjacent non-tumorous liver, and 10 normal liver specimens, dbpA mRNA was quantified by real-time RT-PCR, and promoter methylation was examined by methylation-specific polymerase chain reaction and bisulfite sequencing. The results showed that dbpA mRNA expression levels were higher in HCC compared to corresponding non-tumor tissues (P<0.01) and higher in non-virus-associated HCC compared to virus-associated cases (P<0.01). dbpA promoter was methylated in 37.7% of HCC samples and the promoter methylation was significantly correlated with the low expression of dbpA in non-virus-associated HCC (P<0.01), but not in virus-associated HCC. Surprisingly, poor prognosis was more significantly associated with high dbpA expression in non-tumorous liver (P=0.018) but not with that in HCC. Non-tumorous tissues consist of chronic hepatitis or liver cirrhosis, and these conditions are the background of hepatocarcinogenesis, defined as the hypercarcinogenic state. Our results suggest that the high expression of dbpA in the hypercarcinogenic state is an indicator of poor prognosis.

Correspondence to: Dr Mahmut Yasen, Department of Hepato-Biliary-Pancreatic Surgery, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8510, Japan
E-mail: mahmut@bioinfo.tmd.ac.jp

Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer in the world and the 5-year survival rate of individuals with HCC is lower in 10%, because of its poor prognosis (1,2). It is particularly common in China, Japan, Africa and is also recently increasing in Western countries and USA (3-5). The etiological factors for HCC are well-defined. Hepatitis B virus and hepatitis C virus infection, toxicant exposure, cigarette smoking, alcohol consumption and also metabolic diseases contribute well-documented risk factors for the development of HCC (6,7). In addition, numerous genetic abnormalities associated with the development of HCC have been described. However, the detailed molecular mechanism for the development and progression of HCC is not clear yet (8).

As for other tumors, development of HCC is due to a multistep process with accumulation of genetic and epigenetic alteration in regulatory genes (9-14). CpG Island may be a fundamental contributor to carcinogenesis and cancer progression (15-17). Such epigenetic defects also have been observed in non-cancerous liver tissue of HCC patients. The aberrant methylation observed in HCC may be a consequence of the normal aging process, persistent viral infection or chronic inflammation (18).

DNA binding protein A (dbpA) belongs to the Y-box binding protein family. It is located on chromosome 12 and the molecular weight of dbpA protein is approximately 60 kDa. The cold-shock domain, which is highly conserved from bacteria to human and contain about 70 amino acid residues, is encoded in exons 2-5. It was found that exon 6, which encoded 69 amino acids, is alternatively spliced (19). Previously, we reported dbpA as a candidate molecule that can accelerate inflammation-induced hepatocarcinogenesis. Expression of dbpA protein is associated with the advanced steges of HCC and its nuclear localization is a marker of poor prognosis (20-22).

Most of HCCs arise from chronic hepatitis or live cirrhosis and we consider these situations to be a hypercarcinogenic state (23,24). Our previous study showed that the expression of dbpA was significantly decreased in adjacent non-tumorous

Table I. Clinicopathological features of 96 patients with primary HCC.

Clinicopathological factor	HBV (n=15)	HCV (n=41)	NBNC (n=40)
Age (years, mean ± SD)	54.0±12.3	63.7±7.6	72.3±7.9
Gender (male/female)	11/4	32/9	31/9
AST (IU/l, mean ± SD)	38.7±27.1	54.7±37.5	34.5±26.8
ALT (IU/l, mean ± SD)	43.0±56.9	46.3±50.1	29.5±26.9
Plt (x10^9/l, mean ± SD)	11.0±15.4	11.7±5.56	17.2±8.93
ICG-R15 (%, mean ± SD)	14.6±6.20	16.2±9.91	17.6±10.7
PT% (mean ± SD)	74.4±24.4	85.1±15.4	86.0±11.9
T.bil (mg/dl, mean ± SD)	0.78±0.24	0.84±0.29	0.80±0.39
Alb (g/dl, mean ± SD)	4.20±0.51	3.80±0.40	3.94±0.42
AFP (ng/ml, log10)	2.58±1.50	1.55±1.26	0.99±1.06
PIVKA-II (mAU/ml, log10)	2.32±1.00	2.00±1.08	2.05±1.35
Tumor max size (mean ± SD)	4.75±4.11	3.85±2.22	5.20±3.80
Tumor size (≥2.1 cm)	1/14	5/36	4/36
Tumor size (≥5.0 cm)	10/5	33/8	19/21
No. of tumors (A/B/C/D)	11/3/1/0	23/9/5/4	28/5/3/4
Single/Multiple	11/4	24/17	28/12
Macro type (SN/SNEG/CM/Other)	4/2/7/2	20/12/5/4	19/12/8/1
Histological differentiation (Well/Moderate/Poor)	2/10/3	11/26/4	9/24/7
Growth pattern (Expansive/Invasive)	8/7	33/8	38/2
Capsular formation (-/+)	7/8	4/37	9/31
Capsular invasion (-/+)	7/8	17/24	17/23
Portal vein invasion (-/+)	4/11	23/18	23/17
Micro vascular invasion (-/+)	4/6	23/11	23/11
Macro vascular invasion (-/+)	10/5	34/7	34/6
Recurrence (Abcent/Present)	11/4	25/16	26/14
Stages (I/II/III/IV)	1/4/8/2	2/20/13/6	1/20/13/6

AST, aspartate aminotransferase; ALT, alanine aminotransferase; PLT, platelet; ICG-R15, indocyanine green retention rate at 15 min; PT%, prothrombin time; T.bil, total bilirubin; Alb, albumin; AFP, α-fetoprotein; PIVKAII, protein induced by vitamin K absence or antagonists II; No. of tumors (A/B/C/D): A, 1; B, 2; C, 3; D, ≥4; SN, single nodular type; SNEG, single nodular type with extranodular growth; CM, confluent multinodular type; +, positive; -, negative.

tissues. The regulational mechanism, however, remains unclear. Profile of the CpG sites in the promoter region of dbpA and the correlation with the level of dbpA mRNA expression, methylation status of that promoter region and correlation of clinicopathological feature in HCC are still largely unknown.

In this study, we examined the dbpA mRNA expression level by quantitative real-time reverse transcription PCR in HCC, adjacent non-tumorous tissue and normal liver tissue. We also analyzed the methylation status of promoter CpG island of this gene, in the same tissues and several hepatoma cell lines. The results showed the inverse correlation of the expression level and methylation status, both in cancer tissue and the corresponding non-tumorous tissues. The survival rate was more significantly associated with the low expression level of dbpA in non-tumorous tissues than in HCC tissues.

Materials and methods

Patient characteristics. We analyzed a total of 202 liver subjects, including 96 pairs of HCC and adjacent non-tumorous tissues and 10 normal liver specimens. All patients underwent surgical resection in the Department of Hepato-

Biliary-Pancreatic Surgery at Tokyo Medical and Dental University Hospital between April 2003 and March 2009. This research project was approved by the local ethics committee and all samples were obtained with the patient's informed consent. Tissues were collected during surgery, snap-frozen in liquid nitrogen and then stored at -80˚C for DNA/RNA analysis. A part of the tissue sample was fixed in formalin and embedded in paraffin for histological diagnosis and others analyses. Histological diagnosis was made when two pathologists specializing in liver disease reached the same consensus. Hepatitis B virus (HBV) surface antigen or hepatitis C virus (HCV) antibody was positive in 15 or 41 patients, respectively. Forty patients were negative for both virus markers and there was no patient infected with both. The details of the clinical data from the patients, classified by virus status, are listed in Table I. All normal liver tissues were negative for both viral markers, and histology showed no evidence of fibrosis or inflammation.

DNA and RNA extraction of cell lines and tissue sample. The human hepatoma cell lines, PLC/PRF/5, SK-Hep1 and HepB3 were obtained from the American Type Culture Collection

(Manassas, VA, USA). JHH4, JHH5, HLE, HepG2, Huh1, Huh6 and Huh7 were obtained from the Human Science Research Resources Bank (Osaka, Japan). The conditions of cell culture were reported previously (25). Total RNA was extracted from tissue specimens and human hepatoma cell lines using RNeasy mini kit (Qiagen, Valencia, CA) and treated with RNase-free DNase I according to the manufacturer's instructions. Integrity of RNA was assessed using Agilent Bioanalyzer RNA 6000 Nano Assay (Agilent Technologies, Palo Alto, CA, USA). All samples had RNA integrity number (RIN) >5.0. Total genomic DNA was extracted from cell lines and frozen tissue using a commercial DNA extraction kit (QIAamp DNA Mini Kit; Qiagen) according to the manufacturer's instructions.

Reverse transcription (RT)-polymerase chain reaction (PCR) and real-time quantitative RT-PCR of dbpA mRNA. Two micrograms of total RNA, was reverse-transcribed to cDNA with High-Capacity cDNA Reverse Transcription Kit (Applied Biosytems) using the random primer contained in the kit, following the manufacturer's directions. Quantitative PCR was performed using the SYBR-Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) on the AB 7500 Fast Real-Time PCR System (Applied Biosystems) under the following conditions: 2 min at 50°C, 10 min at 95°C; 40 cycles of 15 sec at 95°C and 60 sec at 60°C. A 171-bp fragment of dbpA cDNA was amplified with 25-mer sense primer in exon 5 (5'-ATGGAGTTCCTGTGGAAGGGAGTCG-3') and antisense primer in exon 6 (5'-CAGAGAACTGCCTATCAGTGGCAGG-3'). For glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 18-mer sense primer (5'-AGCCACATCGCTCAGACA-3') and 19-mer antisense primer (5'-GCCCAATACGACCAAATCC-3') were used. Immediately after the amplification, melting curve protocols were performed to ensure primer-dimers and other non-specific products had been minimized or eliminated. GAPDH transcript was tested as an endogenous reference to calculate the relative expression levels of target genes according to instructions of Applied Biosystems. For the quantitative analysis of specific mRNA expression, CT values were calculated using the 7500 SDS software. The PCR products were separated by gel electrophoresis and the DNA bands were visualized under ultraviolet light for photographing.

Cell culture and 5-Aza-2-deoxycytidine treatment. Hepatoma cell lines were maintained in Dulbecco's minimum essential medium (DMEM; Sigma, St. Louis, MO, USA) containing non-essential amino acid (Invitrogen, Carlsbad, CA, USA) and 10% heat-inactivated fetal bovine serum (FBS, JRH Bioscience, Lenexa, KS, USA); and grown at 37°C in 5% CO_2. After a 48-h incubation in normal medium, cell lines were treated with a demethylating agent, 5 μM 5-Aza-2-deoxycytidine (5-Aza-CdR; Sigma) for the following 3 days, and the medium and the drug were replaced every 24 h. As a control, cell lines were cultured in normal medium which was replaced every 24 h.

Sodium bisulfite modification, sequencing and methylation-specific PCR. Bisulfite modification of extracted DNA (2 μg) was performed using the EpiTect® Bisulfite Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. We searched CpG islands in the dbpA promoter region

(from -500 to +500 bp) (GenBank accession no. AH003216) using CpG Island searcher (http://www.cpgislands.com) with the default setting (% GC11 >55%, ObsCpG/ExpCpG >0.65). The primers were designed by Methyl Primer Express v1.0 software (Applied Biosytems). Sodium bisulfite-treated DNA was amplified by FastStart HiFi PCR System (Roche Applied Science, Germany) for region (-258 to +178) of dbpA gene. The primers were BSF1 (5'-GGGGATCCTGGTTTTTTTTGGTTTTGTAAG-3'), BSR1 (5'-GGAAGCTTTCCTCTACTCTCGCTCAAAC-3'), BSF2 (5'-GGGGATCCTTGAGGGAGGAGAGTTTAATGTATTT-3') and BSR25 (5'-GGAAGCTTCCCTAAACCTCTCATTAACATTTAAAA-3').

The PCR conditions were as follows: 2 min at 95°C, 35 cycles of 30 sec at 95°C, 30 sec at 58°C and 1 min at 72°C, and final 72°C for 3 min. The PCR products were then subcloned into the basic pBluescript II SK (+) vector. Then, we picked 10 independent colonies for each case, determined the sequence of the promoter in each plasmid by ABI PRISM 310 v3.0 Sequence analyzer and examined the extent of methylation by counting the number of methylated cytosine residues.

The methylation status of the dbpA promoter in hepatoma cell lines, 61 paired HCC and normal liver specimens was examined by methylation-specific PCR according to Herman *et al* (26). The first 174 bp containing 17 CpG sites was amplified with a pair of methylation-specific (forward, 5'-GTTTTGTAAGCGATTCGC-3'; reverse, 5'-AAATTTTTCTAAACGACGCA-3') or non-methylation-specific (forward, 5'-TTGGTTTTGTAAGTGATTTGT-3'; reverse, 5'-AAATTTTTCTAAACAACACACCA-3') primer sets. The second 170 bp containing 28 CpG sites was amplified with the methylation-specific forward primer, 5'-AGCGAGGAGTTTAAGGAGC-3'; the reverse primer, 5'-TCGATAACGATTAATCGACG-3'; the unmethylation-specific forward primer, 5'-GAGTGAGGAGTTTAAGGAGT-3' and the reverse primer 5'-CTCAATAACAATTAATCAACA-3'. After heating at 95°C for 10 min, PCR was performed with FastStart HiFi PCR System containing 2.5 U of FastStart High Fidelity Enzyme Blend and in a thermal cycler (GeneAmp PCR System 9700; Applied Biosystems) for 35 cycles, each consisted of denaturation at 95°C for 15 sec, annealing at 58°C for 60 sec and extension at 72°C for 30 sec and final 3-min extension at 72°C. The PCR products were electrophoresed on 2% agarose gels.

Statistical analyses of clinicopathological correlation. Statistical analyses were performed using PASW Statistics 18 software for Windows (IBM). Difference in mRNA levels between groups, were analyzed using the Student's t-test. Fisher's exact test was performed to estimate the significance of gene expression differences between categorical and non-categorical data for each group. The overall survival curve and disease-free survival rate was calculated by the Kaplan-Meier method and rates were reported with 95% confidence intervals. Differences were tested for significance using the log-rank test. P<0.05 was deemed to be statistically significant.

Results

Level of mRNA for dbpA in HCC and adjacent non-tumor tissues. A total of 202 qRT-PCR data was normalized with expression of control gene and the estimated gene expression
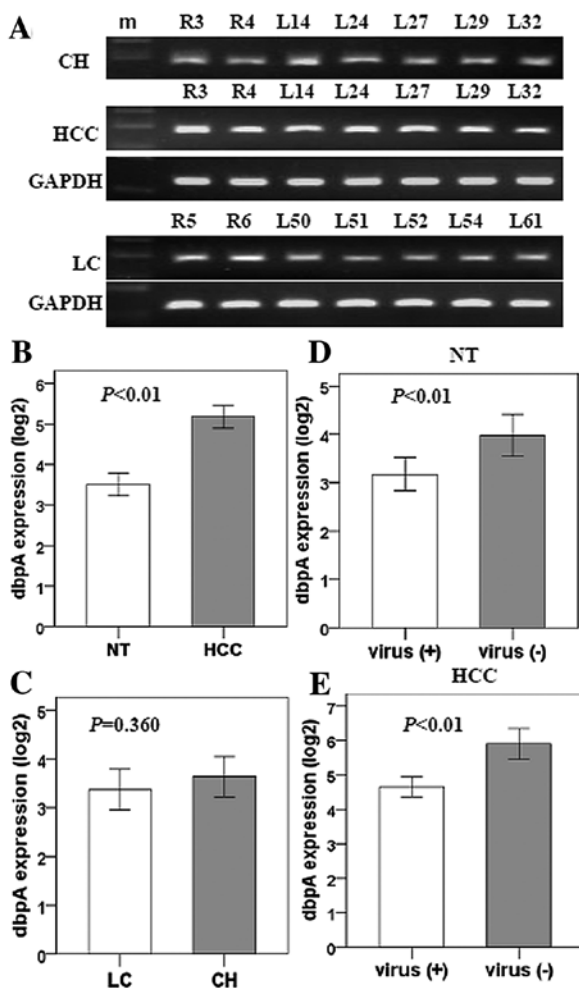
Figure 1. RT-PCR and qRT-PCR analysis of dbpA mRNA in HCC and non-cancerous liver specimens. (A) RT-PCR products were detected in agorose gel electrophoreses. (B) mRNA expression levels of dbpA were compared between HCC and adjacent non-cancerous tissues (NT), (C) between chronic hepatitis and cirrhosis, (D) between virus associated and non-associated NTs, (E) between virus associated and non-associated HCCs. NT, non-tumor; HCC, hepatocellular carcinoma; CH, chronic hepatitis; LC, cirrhosis.

levels were log2-transformed. After the quantitative PCR, the results of the agarose gel electrophoresis confirmed that specific product about 171 bp and no non-specific products were obtained upon amplification of dbpA (Fig. 1A). The differences in mRNA expression levels for dbpA were statistically significant between HCC and corresponding non-tumorous liver (5.18±1.36 vs. 3.51±1.36; P<0.01) (Fig. 1B), There were no statistical significance between chronic hepatitis and cirrhosis (3.63±1.45 vs. 3.37±1.28; P=0.360) (Fig. 1C). dbpA mRNA was more highly expressed in the virus-negative cases than those associated with HBV or HCV infection, both in HCC (5.91±1.41 vs. 4.66±1.06; P<0.01) (Fig. 1E) and adjacent non-tumorous tissue (3.98±1.35 vs. 3.18±1.27; P<0.01) (Fig. 1D). There were no statistical significance between HBV and HCV backgrounds in non-tumorous liver (2.66±1.47 vs. 3.37±1.12; P=0.106) or HCC (4.66±0.76 vs. 4.66±1.16; P=0.959), respectively.

Overexpression of dbpA in non-cancerous tissues is associated with poor prognosis in patients with HCC. The correlations of

dbpA mRNA expression with clinicopathological characters were analyzed both in HCC and the adjacent non-tumorous tissues of 96 HCC patients. Depending on the expression level of dbpA mRNA, the tissue samples were divided into two groups, H or L; H group showing higher mRNA level than the average and L group lower than that. As shown in Table II, there was no statistically significant difference of dbpA expression levels between each categorical clinicopathological group such as age, gender, size of tumor, vessel invasion, stages and others except viral backgrounds. High dbpA expression level was significantly associated with low serum level of protein induced by vitamin K absence or antagonist-II (PIVKAII) (P=0.040).

We further explored whether the expression of dbpA mRNA levels was associated with the survival rates of HCC patients. All patients were followed up until the end-point of the research. Median of the follow-up period was 12.3 months (range, 0.1-59.2 months) and the result showed 23 of 96 patients died. High dbpA expression was associated with the poor prognosis both in overall- and recurrence-free survival rates. This tendency was more obvious and statistically significant in the patients whose non-tumorous tissues showed the high dbpA expression (Fig. 2A and B). The patients with high dbpA expression in HCC tissues also showed poor prognosis, but the difference was not statistically significant (Fig. 2C and D).

Induction of dbpA gene expression by 5-Aza-dC. The expression level of dbpA mRNA was evaluated in 13 HCC cell lines. Quantitative real-time RT-PCR analysis showed that there was higher mRNA expression of dbpA in 9 of 13 HCC cell lines (Fig. 3A). Similar results were also observed in Western blot analysis (data not shown). To verify the role of DNA methylation in the regulation of dbpA expression 8 hepatoma cell lines (JHH1, JHH2, JHH4, HLE, HLF, HepG2, Hep3B and Huh7) were treated with 5-Aza-dC, and then dbpA expression was examined (Fig. 3B). In 6 of 8 cell lines, dbpA expression was significantly up-regulated by 5-Aza-dc treatment (1.4- to 10.1-fold difference) (Fig. 3C), which indicated that silencing of dbpA expression was through hypermethylation of dbpA gene promoter in certain HCC cells.

DNA methylation profile of the CpG islands in the dbpA promoter region. The search for CpG Islands in the dbpA promoter region (Fig. 4A) indicated that the main CpG sites were located in the -258 to -133 region upstream of the transcription start site and +33 to +178 downstream of the transcription start site. The typical 55 CpG sites were located in this region (Fig. 4B). To examine the correlation between DNA methylation and dbpA expression, we further investigated the methylation status of dbpA promoter region in cell lines and liver tissues by bisulfite genomic sequencing shown in Fig. 4C.

Methylation analyses of dbpA promoter in HCC and adjacent non-tumor tissues. MS-PCR was done in 61 paired samples of HCC patients and 10 normal liver specimens. Methylation of CpG island was detected in 23 out of 61 (37.7%) HCC tissues, 30 out of 61 (49.2%) adjacent non-tumor and not detected in any of the normal liver tissues (representative data shown in Fig. 4D). Then, we analyzed the relationship of mRNA levels and methylation status of dbpA in HCC and non-tumor tissues.

Table II. dbpA expression and clinicopathological findings in HCC patients.

| Clinicopathological factor | dbpA expression | | | | | |
| | Non-cancerous | | | HCC | | |
	Low	High	P-value	Low	High	P-value
Age (years)						
<60	13	7	0.130	11	9	1.000
≥60	33	43		41	35	
Gender						
Male	32	42	0.144	40	34	1.000
Female	14	8		12	10	
No. of tumor						
Solitary	30	33	1.000	34	29	1.000
Multiple	16	17		18	15	
Virus infection						
Negative	14	26	0.039[a]	15	25	0.007[a]
Positive	32	24		37	19	
Child-Pugh classification						
A	36	45	0.160	42	39	0.400
B	10	5		10	5	
Tumor size (cm)						
<5.0	31	31	0.671	33	29	0.833
≥5.0	15	19		19	15	
AFP (ng/ml)						
<400	38	40	0.798	41	37	0.604
≥400	8	10		11	7	
PIVKAII (mAU/ml)						
<100	20	25	0.546	19	26	0.040[a]
≥100	26	25		33	18	
Macro type of tumor						
SN	21	22	0.527	23	20	0.758
SNEG	13	13		13	13	
CM	7	13		13	7	
Degree of differentiation						
Well	11	11	0.359	11	11	0.523
Moderate	26	34		35	25	
Poor	9	5		6	8	
Portal vein invasion						
Absent	27	23	0.228	26	24	0.686
Present	19	27		26	20	
Formation of capsule						
pfc (-)	9	11	0.806	7	13	0.077
pfc (+)	37	39		45	31	
Infiltration to capsule						
pfc-inf (-)	18	23	0.540	21	20	0.681
pfc-inf (+)	28	27		31	24	
TNM stage						
I+II	26	22	0.307	26	22	1.000
III+IV	20	28		26	22	
Recurrence of tumor						
Absent	32	28	0.208	36	24	0.147
Present	14	22		16	20	

AFP, α-fetoprotein; PIVKAII, protein induced by vitamin K absence or antagonists II; SN, single nodular type; SNEG, single nodular type with extranodular growth; CM, confluent multinodular type. [a]P<0.05.
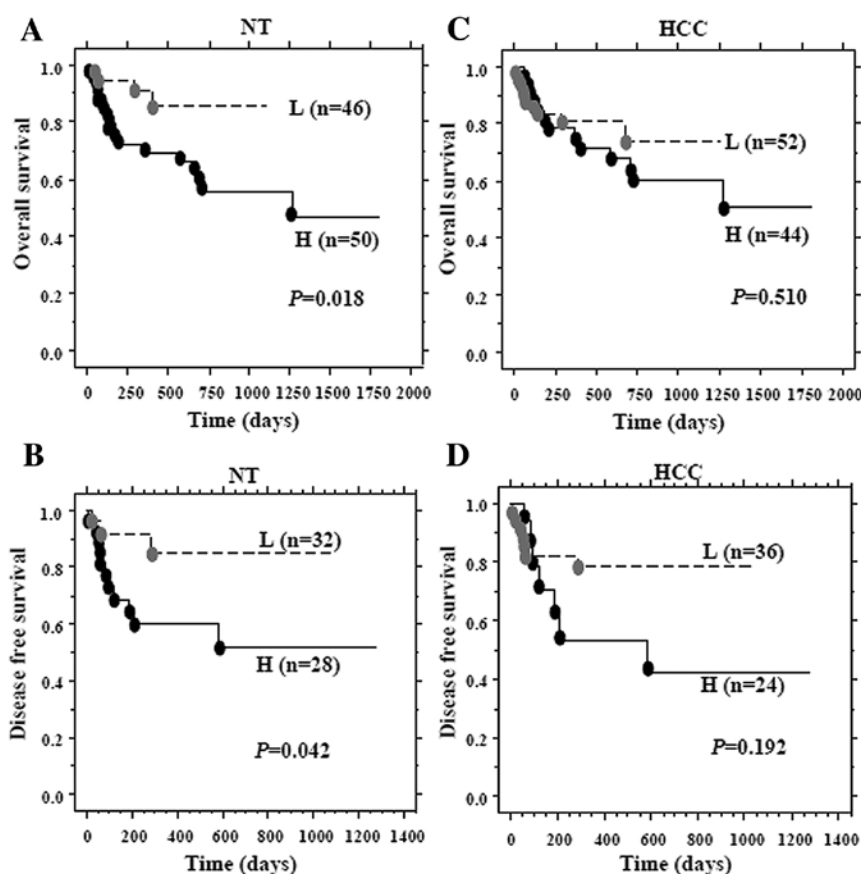
Figure 2. Cumulative overall survival curves (A, C) and tumor recurrence-free survival curves (B, D) of HCC patients after curative resection. Effect of dbpA expression level (High vs. Low) in NT (A, B) or in HCC (C, D) for survival curves are shown. Survival rate of patients with High or Low dbpA expression are indicated by solid or dotted lines, respectively.
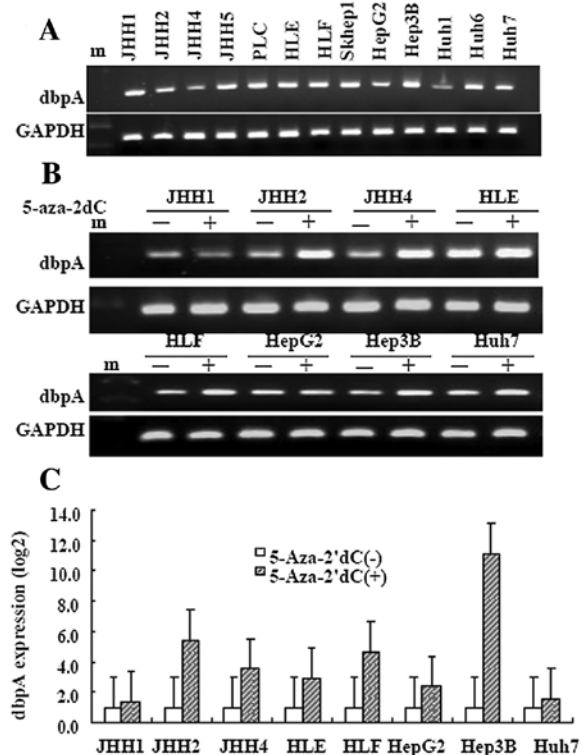

Figure 3. dbpA expression in HCC cell lines. The expression of dbpA mRNA in hepatoma cell lines (A) and effect of 5-Aza-2'dC on dbpA expression (B) are shown. (C) dbpA mRNA was quantified by qRT-PCR.

In HCC, dbpA mRNA levels were significantly higher in the samples with unmethylated promoter than in those with methylated one (5.02±1.01 vs. 6.11±1.37; P<0.01). In non-tumorous samples, there was similar tendency but the difference was not statistically significant (3.65±1.33 vs. 4.10±1.34; P=0.194). Next we analyzed viral or non-viral related patients that the expression levels of dbpA and the methylation status. The results show (Fig. 5D) that the expression more significantly correlated with non-viral related HCC than viral HCC (5.05±1.13 vs. 6.67±1.26; P=0.001) patients in the methylation or unmethylation status, respectively. Fig. 5E shows the representative data of dbpA mRNA expression in the samples with the methylated promoter and those with the unmethylated promoter. Table III shows the association of dbpA promoter methylation and clinicopathological characteristics in 61 HCC patients. Methylated dbpA promoter in the non-tumorous part of HCC was more frequently observed in patients with low serum level of AFP (<400 ng/ml) than patients with high serum level of AFP (≥400 ng/ml) (57.1 vs. 16.7%; P=0.022). Methylated dbpA promoter in HCC tissue was more frequently observed in female than male patients (73.3 vs. 26.1%; P=0.002). Poorly differentiated HCC (88.9%; P=0.041), and low serum level of PIVKAII (<100 mAU/ml) (76.7%; P=0.034) were also associated with the demethylated dbpA promoter in HCC tissues. There was no association between dbpA promoter methylation and age, tumor size, vascular invasion, stages, recurrence of tumor and other factors.
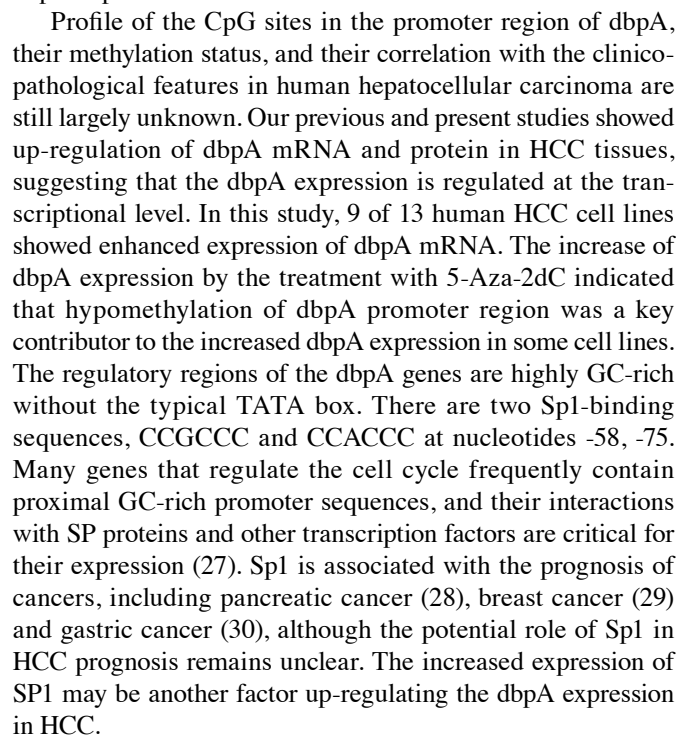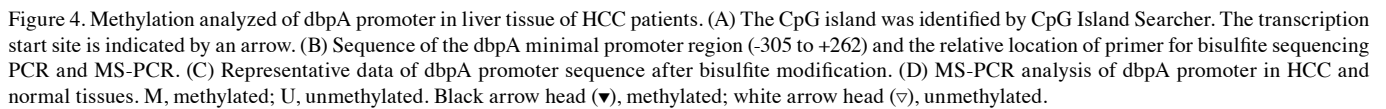
Figure 4. Methylation analyzed of dbpA promoter in liver tissue of HCC patients. (A) The CpG island was identified by CpG Island Searcher. The transcription start site is indicated by an arrow. (B) Sequence of the dbpA minimal promoter region (-305 to +262) and the relative location of primer for bisulfite sequencing PCR and MS-PCR. (C) Representative data of dbpA promoter sequence after bisulfite modification. (D) MS-PCR analysis of dbpA promoter in HCC and normal tissues. M, methylated; U, unmethylated. Black arrow head (▼), methylated; white arrow head (▽), unmethylated.

Figure 5. Relationship of dbpA mRNA expression and the promoter methylation status in NT (A, C) and HCC (B, D). The results of samples including viral cases (A, B) and non-viral cases (C, D) are shown. (E) The methylation or unmethylation of patient mRNA expression levels were detected in agarose gel electrophoresis.

Discussion

We showed that the expression of dbpA mRNA was higher in HCC tissue than in the adjacent non-cancerous part. Unexpectedly, the high dbpA expression level in non-cancerous tissues, but not in HCC, was associated with the poor prognosis of patients (Fig. 2A and B). This result is compatible with our previous study reporting no association of dbpA protein expression in HCC tissue with prognosis of patients, although the nuclear localization of dbpA protein was related to poor prognosis (22). At that time we were not able to analyze the dbpA expression in the non-cancerous tissues.

Profile of the CpG sites in the promoter region of dbpA, their methylation status, and their correlation with the clinico-pathological features in human hepatocellular carcinoma are still largely unknown. Our previous and present studies showed up-regulation of dbpA mRNA and protein in HCC tissues, suggesting that the dbpA expression is regulated at the transcriptional level. In this study, 9 of 13 human HCC cell lines showed enhanced expression of dbpA mRNA. The increase of dbpA expression by the treatment with 5-Aza-2dC indicated that hypomethylation of dbpA promoter region was a key contributor to the increased dbpA expression in some cell lines. The regulatory regions of the dbpA genes are highly GC-rich without the typical TATA box. There are two Sp1-binding sequences, CCGCCC and CCACCC at nucleotides -58, -75. Many genes that regulate the cell cycle frequently contain proximal GC-rich promoter sequences, and their interactions with SP proteins and other transcription factors are critical for their expression (27). Sp1 is associated with the prognosis of cancers, including pancreatic cancer (28), breast cancer (29) and gastric cancer (30), although the potential role of Sp1 in HCC prognosis remains unclear. The increased expression of SP1 may be another factor up-regulating the dbpA expression in HCC.

Table III. Association between methylation status of dbpA and clinicopathological parameters in HCC.

Characteristics	N	Non-cancerous M	UM	P-value	HCC M	UM	P-value
Age (years)							
<60	10	4	6	0.731	4	6	1.000
≥60	51	26	25		19	32	
Gender							
Male	46	20	26	0.146	12	34	0.002[a]
Female	15	10	5		11	4	
No. of tumors							
Solitary	40	20	20	1.000	13	27	0.277
Multiple	21	10	11		10	11	
Tumor size (cm)							
<5.0	39	19	20	1.000	13	26	0.415
≥5.0	22	11	11		10	12	
AFP (ng/ml)							
<400	49	28	21	0.022[a]	17	32	0.342
≥400	12	2	10		6	6	
PIVKAII (mAU/ml)							
<100	30	14	16	0.800	7	23	0.034[a]
≥100	31	16	15		16	15	
Differentiation							
Well	15	9	6	0.504	9	6	0.041[a]
Moderate	37	16	21		12	25	
Poor	9	5	4		1	8	
Portal vein invasion							
Absent	34	17	17	1.000	14	20	0.601
Present	27	13	14		9	18	
Hepatic vein invasion							
Absent	53	26	27	1.000	19	34	0.461
Present	8	4	4		4	4	
TNM stage							
I+II	32	16	16	1.000	12	20	1.000
III+IV	29	14	15		11	18	
Recurrence							
Absent	36	18	18	1.000	14	22	1.000
Present	25	12	13		9	16	

NT, adjacent non-tumor tissue; HCC, hepatocellular carcinoma; AFP, α-fetoprotein; PIVKAII, protein induced by vitamin K absence or antagonists II; M, methylation; UM, unmethylation. [a]$P<0.05$.

We studied the effect of HBV/HCV infection on the dbpA mRNA expression and promoter methylation. The dbpA expression level was higher in virus negative cases than in positive cases, both in NT and HCC parts (P<0.01; Fig. 1D and E). There was no difference in the dbpA expression level between HBV and HCV positive cases (data not shown). In virus-negative cases, the dbpA expression level in HCC was lower in the cases with methylated dbpA promoter than those with unmethylated one (P<0.01; Fig. 5D). These data revealed that the effect of dbpA promoter hypomethylation on the enhanced expression is more obvious in the virus negative cases than in the positive ones. At present we cannot explain these findings, but there are several possibilities. First, in HBV/HCV positive cases, the mechanisms other than promoter methylation may be regulating the dbpA expression. Second, in HBV/HCV negative cases, another unknown carcinogenic factor is acting on the promoter demethylation and enhanced expression of dbpA. Further study is required to verify these possibilities.

The methylated dbpA promoter in non-tumorous parts, but not in HCC parts was associated with low serum level of α-fetoprotein (Table II). The significant association of dbpA

methylation and AFP levels suggests that dbpA methylation status might have potential as a diagnostic marker in HCC. The up-regulation of dbpA and renewed AFP expression in HCC may be the common consequence of tumorigenesis and progression. In the promoter region of α-fetoprotein there are two 'inverted CCAAT', the core sequence of Y-box. However, we do not have the functional data to link AFP expression and that the dbpA could positively regulate the expression of the α-fetoprotein gene. Further study is necessary to clarify the AFP expression and methylation of dbpA, and global analysis will be of increasing importance in the identification of novel hypomethylated genes in HCC.

Regardless of etiology, most of HCC occurs through chronic hepatitis or liver cirrhosis, and these situations are considered to be hypercarcinogenic states (23,24). In hypercarcinogenic states, inflammation-induced genetic and epigenetic changes are accumulated to promote hepatocarcinogenesis. Our study showed that the high dbpA expression in non-cancerous tissue was associated with the poor prognosis of patients and the results suggested that the demethylation related up-regulation of dbpA in hypercarcinogenic states may be accelerating hepatocarcinogenesis. The dbpA expression in chronic hepatitis or liver cirrhosis could be a marker to predict the occurrence of HCC or the prognosis of HCC patients.

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