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Original Article

Hepatitis B virus infection in hepatocellular carcinoma tissues upregulates expression of DNA methyltransferases

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Abstract: Purpose: Our previous research identified that Hepatitis B virus (HBV) infection results in the increased methylation of p16; however, the mechanism(s) of the methylation changes observed following HBV infection are yet to be deduced. DNA methylation is governed by the interaction of DNA methyltransferases (DNMT). To investigate the expression of DNMT in cancerous tissue, cirrhotic tissues and non-cancerous tissue, we examined the relationship between HBV infection and DNMT expression. Methods: We compared the mRNA expression levels of the four DNMTs in cancerous, cirrhotic and matched non-cancerous tissues of HCC with HBV infection by real-time PCR. Results: The results showed that compared with the level in the corresponding non-cancerous liver tissues, the levels of DNMT1, DNMT3A and DNMT3B were elevated in 54.5%, 68.2% and 38.6% of cancerous tissues and 31.4%, 40% and 25.8% of cirrhotic tissues, respectively. The average mRNA expression for DNMT2 in cancerous and cirrhotic tissues of HCC was not significantly different from that in the corresponding non-cancerous liver tissues. In HBV-associated tissue samples, both the average level and the elevated frequency of DNMT1, DNMT3A and DNMT3B mRNA expression were significantly higher than in non-HBV-associated cirrhotic and cancerous tissues; even in non-cancerous tissues, the mRNA levels of DNMT1 and DNMT3A in HBV-associated samples were significantly higher than in the non-HBV-associated samples. Correlations analysis demonstrated a significant association between HBV infection and the overexpression of DNMTs and p16 methylation. Conclusions: The results of our current study suggest that persistent HBV infection can stimulate the overexpression of DNMTs, particularly DNMT1, DNMT3A and DNMT3B, which may result in the hyper-methylation/inactivation of p16, thus indirectly regulating the progression of hepatocellular carcinogenesis.

Keywords: Overexpression, DNA methyltransferase, hepatitis B virus, hepatocellular cellular carcinoma

Introduction

The persisting process of hepatocyte damage and regeneration in chronic viral hepatitis or in toxic insult may provoke an uncontrolled growth of hepatocytes and may eventually result in malignant transformation due to a disruption of the cell cycle regulatory mechanisms. An abnormal control of the cell cycle, particularly during G1 to S phase transition, is associated with many types of human carcinogenesis. Among the elements of the complex molecular machinery of G1 phase progression, p16 is a negative regulatory protein that can competitively bind to the cyclin-dependent kinase 4 (Cdk4) protein, resulting in the inhibition of the interaction of Cdk4 with cyclin D1 and subsequent passage through the G1 phase. Inactivation of the p16 gene, leading to the disruption of cell cycle control, has been known to be involved in many types of malignant tumours including hepatocellular carcinoma (HCC) [1-3]. Previous reports show that although the loss or low expression of *p16* is a common phenomenon in HCC, mutations or homozygous deletions in this gene are rare [2, 4]; promoter methylation is thought to be the primary mechanism underlying the inactivation of p16 in HCC [5-7]. A high rate of p16 methylation was reported by Csepregi A. *et al.,*

[3] (69.7%), Zhang C. *et al.,* [4] (62.5%), Form eister EJ *et al.,* [5] (79%) and our previous work [6]. Our results show that in addition to the high rate of p16 methylation found in 31/44 (70.5%) of HCC patients [6], a much higher rate of p16 methylation was detected in the cancerous and cirrhotic tissues of HCC associated with HBV infection. This observation was also reported by other groups [7]. These results signify that HBV infection is a factor promoting methylation of p16, although little is known about the mechanism of HBV-induced methylation of p16.

The process of DNA methylation is known to be governed by the interaction of *trans*-acting enzymes called DNMTs [8, 9]. Human DNMTs, DNMT1, DNMT2, DNMT3A and DNMT3B, have been identified and reported to be able to maintain this DNA methyltransferase activity and/or *de novo* methylase activity. In addition, the over-expression of these DNMTs has been detected in some human malignancies such as carcinomas of pancreatic ductal adenocarcinoma, testicular seminoma, idiopathic thrombocytopenic purpura [10-12]. With respect to hepatocarcinogenesis, the overexpression of different DNMT proteins and mRNA have been reported [13, 14], but their relations with HBV infection status have not been analysed.

Thus, we hypothesized that HBV may promote the hypermethylation of p16, thereby inducing the expression of DNMT. In the present work, to investigate the role of HBV-mediated overexpression of the DNMT mRNA and *p16* methylation in HCC, we examined the DNMT mRNA in 44 cases of cancerous tissues and matched cirrhotic and non-cancerous liver tissues of HCC patients and cell lines with different HBV infection status, tumour stage and differentiation. The relationship between the levels of DNMTs and p16 hypermethylation was also evaluated.

Materials and methods

Cell lines and culture

HepG2 (human hepatoblastoma cell line, ATCC Number: HB-8065) and Hep3B (human hepatocellular carcinoma cell line, ATCC Number: HB-8064) cells were cultured in DMEM with 10% FCS and incubated at 5% CO_2, at 37°C. Cells (2×10^5/ml) were plated on round cover slips measuring 12 mm in diameter and cultured in 24-well culture plates.

Patients and specimens

Following informed consent and ethics approval, 44 cases of tissue specimens from primary HCC and the corresponding cirrhotic and non-cancerous liver tissues were obtained from surgically resected material from 44 patients who were treated at an affiliated hospital. Tumor staging was based on the NCCN Guidelines in Oncology. The specimens were obtained from 35 men and 9 women, of whom 32 cases had HBV infection and 12 cases did not (HCC with HCV infection was excluded in this study). The cirrhotic tissues (> 2 cm distance to the resection margin) were obtained and the non-cancerous tissues (> 5 cm distance to the resection margin) were obtained, respectively. However, only 35 corresponding cirrhotic tissues were collected as the removal of the cirrhotic tissues failed in 9 patients. Each specimen was determined to be HCC or cirrhotic or non-cancerous tissue by pathological examination. The resected tissue was divided into two parts, one of which was frozen immediately after careful separation of the non-cancerous, cirrhotic and cancerous tissue and stored under liquid nitrogen until tissue DNA and total RNA extractions; the remaining tissue was fixed in 10% buffered formaldehyde solution for pathological diagnosis by the department of pathology

RNA extraction and cDNA synthesis

Total RNA was also extracted using TRIzol® Reagent (InterGen Discovery Products, Pur chase, NY, USA) according to the manufacture's protocol. RNA concentration was estimated by spectrophotometric method (BioRad Smart Spec™Plus Spectrophotometer, CA, USA). First-strand cDNA was prepared from total RNA using Promega reverse transcription system (Promega, WI, USA) based on the manufacturer's instructions. cDNA was used immediately or stored at -80°C until use.

Real-time PCR detects mRNA expression of DNMTs

Primer sets used for the polymerase chain reactions (PCR) are shown in (**Table 1**). The PCR reactions were performed using the SYBR Green PCR Core Reagents kit (Takara Bio Inc, Shiga, Japan). Real-time detection of the emission intensity of SYBR Green bound to double-stranded DNAs was performed using the ABI

Table 1. Primer sets used for real time PCR

Gene	Primer	Gene No	Size
DNMT1	5-CGACTACATCAAAGGCAGCAACCTG-3 5-TGGAGTGGACTTGTGGGTGTTCTC-3	NM001379	166
DNMT2	5-AAGCTGTAAGCCAGCCCATATAC-3 5-TCAGCAGTGAACAGAACCTACATG-3	AJ223333	148
DNMT3A	5-CGAGTCCAACCCTGTGATGATTG-3 5-GCTGGTCTTTGCCCTGCTTTATG-3	AF067972	221
DNMT3B	5-TTGGAATAGGGGACCTCGTGTG-3 5-AGAGACCTCGGAGAACTTGCCATC-3	NM066892	152
GAPDH	5-GAAGGTGAAGGTCGGAGTC-3 5-GAAGATGGTGATGGGATTTC-3	NM002046	226
Histone 4	5-CAACATTCAGGGCATCACCAA-3 5-CCCGAATCACATTCTCCAAGAA-3	M16707	131

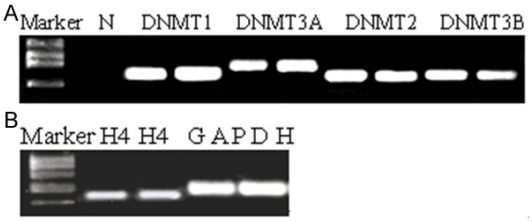

Figure 1. Expression of mRNA for DNMT1, DNMT2 DNMT3A and DNMT3B in HCC tissues. Expression of mRNA for DNMT1, DNMT2 DNMT3A and DNMT3B were assayed by real-time RT-PCR. The signal intensity in the figure is not directly proportional to the amount of the template RNA in each sample, because electrophoresis was performed after 40 PCR cycles. A: DNMT1, 2, 3 A and 3 B; B: Reference gene H4 and GAPDH.

7000 Sequence Detection System (ABI Biosystems). The quantitative PCR reactions were performed in triplicate for each sample-primer set, and the mean of the three experiments was used as the relative quantification value. To accurately determine the starting copy number regardless of the precise amount and qualities of input DNA, we also quantified internal control genes (GAPDH and Histone 4) in each single reaction and normalized the target genes to them. The ABI7000 sequence detecting system software automatically determines the C_t value and refers the starting copy numbers in each sample. The PCR reaction mixture (27 μl) included approximately 50 ng DNA, 18 pmol primer, 2.0 mM $MgCl_2$, 0.2 mM dNTP, 0.5 μl 20 × SYBR Green and 1.5 Unit of Taq polymerase in 1 × PCR buffer. For diminishing noise signal of primer dimer, the thermal profiles were modified according to Ball's method by altering the PCR strategy to take advantage of the ABI 7000's ability to measure fluorescence at a temperature greater than the melting point of primer dimers. The resulting measurements determine fluorescence of only the desired PCR product and can eliminate the fluorescence induced by primer dimers and obtain accurate product quantitation.

A total of 40 cycles were used; at the end point of 40 PCR cycles, dissociation curve analysis was performed, the reaction products were separated electrophoretically on a 3% agarose gel and stained with ethidium bromide for further confirmation of the PCR products. The level of mRNA for the DNMTs was expressed as a ratio relative to the GAPDH and histone H4 mRNA in each sample. The levels of mRNA for each DNMTs in cancerous and cirrhotic tissues were compared with the mean level in the corresponding non-cancerous tissue; the level of mRNA of DNMTs from HCC with HBV infection was compared with those without HBV infection.

Detection of markers associated with HBV infection in blood

Blood samples were centrifuged at 5000 rpm, plasma was carefully collected from the EDTA-containing plain tubes and transferred into sample tube of the AxSYM system for analysing HBV markers, including HBsAg, HBsAb, HBeAg, HBeAb, HBcAb and HCV antibody.

Statistical analysis

ANOVA was used for analysing the differences in overexpression of DNMTs among three or more subgroups, for instance, different tissues, infection status of HBV and different tumour differentiation and stage (SPSS15.0 software). T test was used for analysing the differences in overexpression of DNMTs between two groups. Non-parametric correlation and logistic regression analysis were applied to the correlation between DNMT mRNA expression and p16 methylation, clinical pathological data, such as viral status, gender, tumour differentiation and staging.

Results

mRNA expression of DNMTs in all samples

Following quantitative PCR, examination of the agarose gels confirmed that specific products

of approximately 166 bp, 148 bp, 221 bp, 152 bp, 226 bp and 131 bp were obtained upon amplification of DNMT1, DNMT2, DNMT3A, DNMT3B, GAPDH and H4, respectively (**Figure 1A** and **1B**).

The mRNA levels of DNMTs in cancerous, cirrhotic and non-cancerous tissues of HCC are presented in (**Table 2**). Overall, the average level of expression of the four kinds of DNMT mRNA were all elevated in cancerous and cirrhotic tissues than in non-cancerous tissues, and at least one kind of DNMT was increased in every case of HCC. However, DNMT mRNA levels were selectively increased in cirrhotic tissues. Some cases showed increased DNMT1 or DNMT2, some showed increased DNMT3A or DNMT3B and some had randomly increased DNMTs among the four kinds of DNMTs. The specific DNMT cases are described in the subsequent section.

The expression of DNMT1 mRNA was elevated in 24/44 (54.5%) of cancerous and 11/35 (31.4%) of cirrhotic tissues compared with the expression in corresponding non-cancerous liver tissues. The average levels of mRNA for DNMT1 in cancerous and cirrhotic tissue of HCC were significantly higher than those in the corresponding non-cancerous liver tissues ($P < 0.01$ for cancerous tissue, $P < 0.05$ for cirrhotic tissue).

The DNMT2 mRNA expression was elevated in 8/44 (18.2%) of cancerous and 4/35 (11.4%) of cirrhotic tissues compared with the level in the corresponding non-cancerous liver tissues. The average level of mRNA for DNMT2 in cancerous tissues of HCC was significantly higher than in the corresponding non-cancerous liver tissues ($P < 0.05$).

The status of DNMT3A was similar to that of DNMT1, with the expression elevated in 30/44 (68.2%) of cancerous and 14/35 (40%) of cirrhotic tissues compared to the corresponding non-cancerous liver tissues (7/35, 20%). The average level of mRNA for DNMT3A in cancerous and cirrhotic tissue was significantly higher than that in the corresponding non-cancerous liver tissues ($P < 0.01$ for cancerous and $P < 0.05$ for non-cancerous tissues).

The expression of DNMT3B mRNA was elevated in 17/44 (38.6%) of cancerous and 9/35 (25.8%) of cirrhotic tissues compared with the level in the corresponding non-cancerous liver tissues. The average expression of DNMT3B mRNA in cancerous tissues was significantly higher than in the corresponding non-cancerous liver tissues ($P < 0.05$).

Overall, from non-cancerous tissues to cancerous tissues, we not only observed a gradual increase in the mRNA expression of DNMTs, but the type of DNMTs also increased from 1 or 2 in non-cancerous tissue to 2 or 3 in cancerous tissue.

mRNA expression of DNMTs in different clinical pathological data of HCC

The expression of the mRNA levels of DNMTs in different clinical pathological data is presented in (**Table 3**). Although gender did not significantly influence the expression of DNMT mRNA, tumour staging and differentiation ($P > 0.05$), an increasing expression of DNMTs was obse rved in males, with poorly differentiated and high stage tumours. The melting curve of DNMTs, GAPDH, H4 were shown in (**Figure 2**).

mRNA expression of DNMTs in all samples with and without HBV infection

Based on the HBV infection status, all samples were divided into two groups: one with HBV infection, which has at least two positive HBV-linked markers and is further subdivided into three subgroups (details in **Table 4**) and the other group without HBV and/or HCV infection.

The detailed data of levels of mRNA for DNMTs in groups with HBV infection and without it were shown in **Table 4**.

The average expression of DNMT1, DNMT3A and DNMT3B mRNA in cirrhotic and cancerous tissues with HBV infection was significantly higher than that without it ($P < 0.01$ for DNMT1 and DNMT3A, $P < 0.05$ for DNMT3B); even in non-cancerous tissues, the mRNA level of DNMT1 and DNMT3A in HBV-associated samples was significantly higher than in the non-HBV-associated samples ($P < 0.05$). The average expression of DNMT2 mRNA in all the tissues ($P > 0.05$) and DNMT3B mRNA in HBV-associated non-cancerous tissues were not found markedly higher than in non-HBV-linked tissues ($P > 0.05$).

Table 2. mRNA expression of DNMTs in cancerous, cirrhotic and non-cancerous tissues of HCC patients

Tissues	n	Average expression levels of mRNA (95% confidence interval)							
		DNMT1		DNMT2		DNMT3A		DNMT3B	
		GAPDH	H4	GAPDH	H4	GAPDH	H4	GAPDH	H4
Cancerous tissue	44	1.06±0.25*	1.02±0.24*	0.70±0.14*	0.68±0.13*	1.04±0.21*	0.99±0.19*	0.53±0.14*	0.51±0.13*
Cirrhotic tissues	35	0.78±0.18**	0.75±0.16**	0.63±0.13	0.62±0.12	0.86±0.19**	0.84±0.18**	0.42±0.12	0.40±0.10
Non-cancerous tissue	44	0.58±0.11	0.55±0.10	0.58±0.12	0.50±0.11	0.57±0.17	0.65±0.18	0.30±0.11	0.28±0.10

Note: *$P < 0.05$; ** $P < 0.01$.

Table 3. mRNA expression of DNMTs in different clinical pathological data in HCC patients

	n	Average expression levels of mRNA (95% confidence interval)							
		DNMT1		DNMT2		DNMT3A		DNMT3B	
		Normalized with		Normalized with		Normalized with		Normalized with	
		GAPDH	H4	GAPDH	H4	GAPDH	H4	GAPDH	H4
Gender									
Male	35	1.15±0.23	1.12±0.20	0.74±0.15	0.73±0.14	1.21±0.23	1.19±0.21	0.54±0.12	0.50±0.10
Female	9	1.04±0.24	1.04±0.23	0.66±0.13	0.65±0.13	0.99±0.21	0.97±0.19	0.44±0.11	0.43±0.09
Differentiation									
Well	20	0.98±0.19	0.95±0.18	0.58±0.16	0.56±0.15	0.96±0.22	0.93±0.19	0.43±0.12	0.41±0.10
Moderate	18	1.20±0.21	1.19±0.19	0.70±0.15	0.68±0.14	1.12±0.18	1.08±0.17	0.46±0.13	0.45±0.12
Poor	6	1.23±0.24	1.23±0.22	0.74±0.16	0.72±0.15	1.20±0.19	1.29±0.18	0.50±0.13	0.48±0.11
Tumor staging									
I	20	1.08±0.19	0.98±0.18	0.65±0.15	0.64±0.15	1.01±0.23	0.98±0.21	0.44±0.12	0.44±0.11
II	15	1.17±0.22	1.15±0.19	0.69±0.14	0.68±0.13	1.12±0.21	1.08±0.19	0.46±0.14	0.45±0.13
III	9	1.25±0.24	1.23±0.21	0.72±0.16	0.71±0.15	1.18±0.20	1.17±0.18	0.49±0.14	0.48±0.12

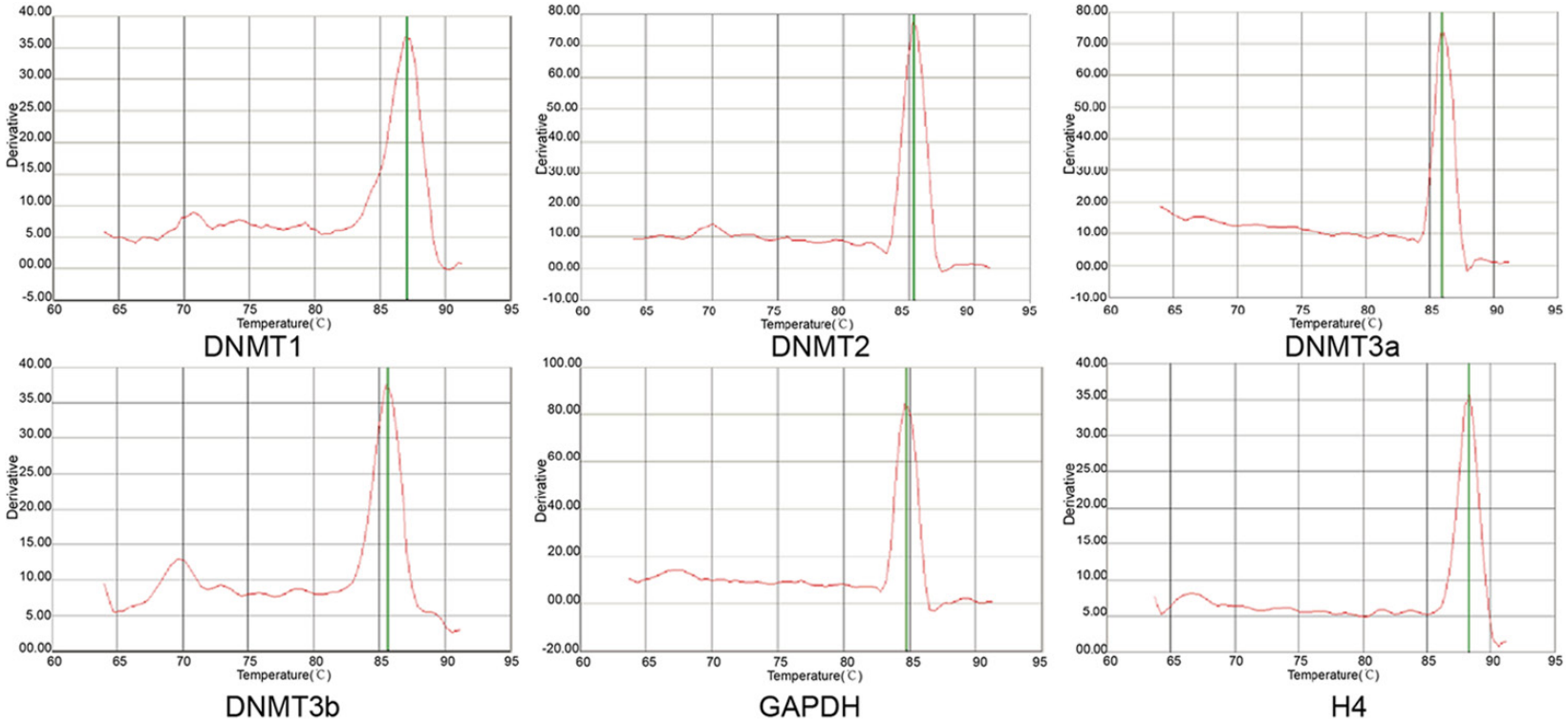

Figure 2. The melting curve of DNMTs, GAPDH, H4

Table 4. mRNA expression of DNMT in cancerous, cirrhotic and non-cancerous tissues of HCC patients with different HBV infection status

Tissues	n	Average expression levels of mRNA (95% confidence interval)							
		DNMT1		DNMT2		DNMT3A		DNMT3B	
		GAPDH	H4	GAPDH	H4	GAPDH	H4	GAPDH	H4
Cancerous tissue	44								
With HBV infection	32	1.22±0.21*	1.14±0.18*	0.75±0.14	0.71±0.13	1.35±0.23*	1.23±0.18*	0.67±0.13**	0.65±0.11**
HBsAg+, HBeAg+	7	1.35±0.26	1.31±0.24	0.86±0.12	0.83±0.11	1.39±0.22	1.37±0.20	0.72±0.14	0.69±0.13
HBsAg+, HBeAg-	18	1.23±0.23	1.20±0.22	0.75±0.13	0.72±0.11	1.34±0.20	1.32±0.19	0.68±0.13	0.66±0.11
Other markers+※	7	1.10±0.17	1.02±0.16	0.68±0.11	0.67±0.10	1.30±0.18	1.29±0.18	0.57±0.11	0.55±0.10
Without HBV infection	12	0.85±0.24	0.83±0.20	0.66±0.11	0.64±0.09	0.94±0.16	0.92±0.15	0.50±0.08	0.47±0.08
Cirrhotic tissue	35								
With HBV infection	24	0.98±0.17*	0.95±0.15*	0.64±0.13	0.63±0.13	0.99±0.19*	0.98±0.18*	0.58±0.15**	0.55±0.14**
HBsAg+, HBeAg+	7	1.00±0.21	0.97±0.19	0.69±0.15	0.68±0.14	1.05±0.20	1.01±0.19	0.63±0.16	0.56±0.15
HBsAg+, HBeAg-	10	0.96±0.17	0.94±0.16	0.65±0.13	0.64±0.13	0.96±0.18	0.94±0.16	0.58±0.14	0.53±0.14
Other markers+*	7	0.91±0.17	0.89±0.15	0.62±0.12	0.61±0.11	0.90±0.18	0.89±0.17	0.54±0.13	0.49±0.12
Without HBV infection	11	0.70±0.14	0.68±0.12	0.60±0.14	0.58±0.13	0.72±0.16	0.70±0.15	0.39±0.12	0.38±0.11
Non-cancerous tissue	44								
With HBV infection	32	0.63±0.14**	0.61±0.12**	0.55±0.12	0.54±0.11	0.66±0.23**	0.64±0.17**	0.34±0.07	0.32±0.06
HBsAg+, HBeAg+	7	0.73±0.16	0.71±0.14	0.60±0.13	0.58±0.12	0.71±0.25	0.71±0.22	0.36±0.12	0.35±0.11
HBsAg+, HBeAg-	18	0.67±0.15	0.65±0.14	0.56±0.12	0.54±0.11	0.65±0.22	0.63±0.21	0.33±0.11	0.33±0.10
Other markers+*	7	0.59±0.13	0.57±0.12	0.48±0.11	0.47±0.10	0.58±0.20	0.56±0.18	0.32±0.10	0.30±0.09
Without HBV infection	12	0.42±0.10	0.41±0.08	0.48±0.10	0.47±0.09	0.44±0.16	0.43±0.16	0.28±0.06	0.26±0.06

Note: 1 ※Including HBsAb, HBeAb and HBcAb, cases with at least two positive of them in serum were included into this group, patients with HCV infection was excluded in this study. 2 *$P < 0.05$; **$P < 0.01$.

The average expression of individual DNMTs and their elevated proportion were significantly higher in HBV-associated tissues. The frequency of elevated mRNA expression of DNMT1 (24/32, 75%), DNMT3A (29/32, 90.63%) and DNMT3B (21/32, 65.63%) in cancerous liver tissues with HBV infection was significantly higher than that in tissues that lacked HBV infection (DNMT1 3/12; DNMT2 25%; DNMT3A 4/12, 33.33%; DNMT3B 3/12, 25%). The situation of DNMT1, DNMT3A and DNMT3B in cirrhotic tissues were similar to those in cancerous tissues (DNMT1, 62.5% vs. 27.27%; DNMT3A 75% vs. 36.67%; DNMT3B 50% vs. 18.18%, HBV infection vs. non-HBV infection). Even in the non-cancerous tissues, an elevated expression of DNMT1 and DNMT3A was observed in the HBV infection group than in the uninfected group: 31.82 % (14/44) vs. 11.36% (5/44) of DNMT1, 45.45% (20/44) vs. 15.91% (7/44) of DNMT3A, respectively.

However, within the HBV-associated subgroups, there were no significant differences in the expression levels of the four kinds of DNMT mRNAs between the various HBV infection statuses, whether positive for HBsAg, HBeAg or other markers ($P > 0.05$).

mRNA expression of DNMTs in Hep3B and HepG2

The analysis of the mRNA expression for DNMT in Hep3B and HepG2 showed that for the expression of DNMT1, DNMT2, DNMT3A and DNMT3B in Hep3B cells were 2.0, 1.2, 3, 1.8 fold higher than in HepG2 cells.

Correlation between mRNA expression of DNMTs, p16 methylation and HBV infection

All cases with p16 hypermethylation present at least one kind of increased DNMT expression in cancerous tissues, 58% (18/31) with two DNMTs and 42% (13/31) with three DNMTs. We found that individuals differ in the combination of high level of DNMT expression, particularly the combination of DNMT1 and DNMT3A or DNMT3B.

Correlation analysis showed a significant relationship between p16 methylation and DNMTs mRNA expression ($P = 0.0013$, 0.025 and 0.041 for DNMT1, DNMT3A and DNMT3B, respectively). In addition, a significant association between HBV infection and overexpression of DNMTs was observed ($P = 0.009$ for DNMT1, 0.006 for DNMT3A, 0.03 for DNMT3B). The relationship between HBV and p16 methylation was presented in our previous report [15].

Discussion

Altered expression of DNMT mRNA has been observed in HCC [15, 16]. However, the role of DNMTs during hepatocellular carcinogenesis is yet unclear. It remains to be determined whether the increased methyltransferase activity of DNMTs is the primary route to DNA hypermethylation of tumour suppressor genes. We have evaluated a role for DNMTs in p16 hypermethylation during hepatocellular carcinogenesis by comparing the altered expression profiles of DNMT mRNAs and the frequency of p16 hypermethylation in HCCs, with and without HBV infection.

The elevated mRNA expression of DNMT1, DNMT3A and DNMT3B was detected in 54.5%, 68.2% and 38.6% of cancerous liver tissues, respectively. Moreover, the frequency of elevated mRNA expression of DNMT1, DNMT3A and DNMT3B in cirrhotic tissues was 31.4%, 40% and 25.8%, respectively. With the exception of DNMT2, the average expression of DNMT1, DNMT3A and DNMT3B mRNA were significantly higher in liver tissues showing cirrhosis and even higher in cancerous tissues than in histologically normal non-cancerous tissues. Com pared with the corresponding non-cancerous tissues, the proportion of elevated DNMTs is also significantly higher in cirrhotic and cancerous tissues. The average expression and frequency of DNMTs mRNA gradually increased from non-cancerous, cirrhotic to cancerous tissues, thus indicating that the increased expression of DNMT mRNA may be an early event and play some role in the development of hepatocarcinogenesis. Interestingly, the increased frequency of expression of DNMT3A in cancerous and cirrhotic tissues was roughly consistent with the p16 methylation rate in the corresponding tissues [15]. This indicates that of the four kinds of DNMTs, DNMT3A may be one of the major contributing factors responsible for p16 methylation during hepatocarcinogenesis. However, because of the cooperative and interactive nature of DNMTs, a role for the other DNMTs in p16 methylation cannot be ruled out.

Both HBV infection and inactivation of key cell cycle regulation genes are important events in the development of HCC. Our previous research shows that p16 methylation is detected at an increasing frequency from chronic hepatitis, cirrhosis (which are considered to be precancerous conditions) to HCC [6]. The incidence of p16 methylation in HCC was higher than in cirrhosis or chronic hepatitis; so p16 CpG islands hypermethylation is an early event during hepatocellular carcinogenesis. In the cancerous, cirrhotic or non-cancerous tissues with HBV infection, the frequency of p16 methylation was higher than in uninfected tissues. Recently, studies by other groups also demonstrated that HBV infection contributes to hypermethylation of tumour suppressor genes [17, 18]. Thus HBV infection can promote the methylation of p16 in HCC. However, the mechanism underlying HBV-induced p16 hypermethylation is still not clear. In our experiments, the various tissue samples (cancerous, cirrhotic and non-cancerous) were divided into two groups based on the HBV infection status. The HBV-infected tissues, which have at least two positive HBV-linked markers, were further divided into three subgroups; the other group was without HBV or HCV infection (no evidence to demonstrate that hepatitis C virus did not influence the expression of DNMTs; samples with HCV infection were eliminated from this study). The mRNA levels of DNMT1, DNMT3A and DNMT3B in HBV-associated cancerous tissues were significantly higher than in uninfected tissues. Most of HCC develops in the background of chronic liver diseases such as chronic viral hepatitis or liver cirrhosis. In the HBV-associated cirrhotic tissues, mRNA levels of DNMT1, DNMT3A and DNMT3B were higher than non-HBV-associated cancerous tissues. Even in non-cancerous tissues, the mRNA levels of DNMT1 and DNMT3A in HBV-associated samples were significantly higher than in non-HBV-associated.

Furthermore, from non-cancerous tissues to cancerous tissues, not only did the mRNA level of DNMTs gradually increase, but also the type of DNMTs increased from two kinds in non-cancerous tissue to three kinds in cancerous tissue. In addition, the frequency of elevated mRNA expression of DNMT1, DNMT3A and DNMT3B in cirrhotic and cancerous tissues with HBV infection also gradually increased and were significantly higher than in uninfected tissues. A significant correlation between HBV infection, p16 methylation and overexpression of mRNA for DNMT1, DNMT3A and DNMT 3B further indicate the association between HBV infection and high expression of mRNA of DNMT and p16 methylation.

To further demonstrate the role of HBV in stimulating the expression of DNMT mRNA, expression of DNMTs mRNA in HBV-associated and non-HBV cell lines were analysed. HepG3B is a cell line of HCC, which contains an integrated hepatitis B virus genome and can secrete HBsAg [19]. HepG2 is a human hepatoblastoma cell line, where no evidence of a Hepatitis B virus genome in this cell line. The analysis results of mRNA levels of DNMTs in HepG2 and HepG3B cell lines showed that the expression of DNMT1, DNMT3A and DNMT3B mRNAs in Hep3B cells was higher than in HepG2 cells, particularly DNMT3A. Although studies published by other groups have shown that it is slightly higher in tumour cell lines than in normal cells, our study demonstrated that it is 3-fold higher in HepG3B than in HepG2 cells. The contradiction may be a result of two reasons: (1) different research subjects; (2) the role of HBV in Hep3B, HBV stimulated the expression of DNMT3A.

All of the results together indicate that the DNMT mRNA expressions increased during the early stage of hepatocarcinogenesis. Both HBV infection and mRNA overexpression of DNMTs are an early event of hepatocarcinogenesis; HBV infection may upregulate the DNMT mRNA expression and subsequently promote the hypermethylation of tumour suppressor genes associated with HBV infection.

Evidence provided by some other group shows that a certain virus and its components can induce the down-regulation of tumour suppression genes via activation of DNMTs. Fang et al., [20] found that the infection of lymphoid cells with integration-defective HIV-1 can increase DNMT1 expression and the methylation in the promoters of genes such as the p16 gene, silencing their expression. The Epstein-Barr virus oncogene product, latent membrane protein 1, could induce the expression and activity of DNMT1, DNMT3A and DNMT3B, resulting in the hypermethylation of the proximal E-cadherin promoter in cultured human nasopharyngeal carcinoma cell lines [21]. In the development of gastric cancer, DNMT1 overexpression correlated

significantly with poorer tumour differentiation and hypermethylation of the *h-MLH, E-cadherin* and *thrombospondin-1* genes and with the methylation phenotype in gastric cancers. DNMT1 overexpression was also associated with EBV infection [22-24]. These results suggest that DNMT1 overexpression may not be just a secondary effect of increased cancer cell proliferative activity, but may be associated with EBV infection and other etiological factors during gastric carcinogenesis. Furthermore, DNMT1 may play a significant role in the development of poorly differentiated gastric cancers by inducing frequent DNA hypermethylation of multiple CpG islands [22]. SV40 T antigen expression results in the elevation of DNMT mRNA, DNMT protein levels and global genomic DNA methylation. A T antigen mutant that has lost the ability to bind pRb does not induce DNMT. Inhibition of DNMTs by antisense oligonucleotide inhibitors results in the inhibition of induction of cellular transformation by T antigen, as determined by a transient transfection and soft agar assay [25]. These results suggest that the elevation of DNMT is an essential component of the oncogenic program induced by T antigen. Recently, Lee et al, [26] found that the HBV component, HBx antigen, could stimulate DNMT1 transcription and over-expression, which induced the hypermethylation of E-cadherin, resulting in the repression of its expression.

The role of the DNA methyltransferases DNMT1, DNMT3A, and DNMT3B in tumorigenesis has been extensively analyzed. In contrast, there are few studies of DNMT2, in human cancers. DNMT2 has been shown to have a prominent tRNA methyltransferase activity in various model systems which separates this enzyme from other DNMT proteins. DNMT2 contains all the catalytic signature motifs of conventional (cytosine-5) DNA methyltransferases, but has comparably low DNA methyltransferase activity. More recently, it was shown that DNMT2 methylates tRNAAsp, which suggested that the substrate specificity of the enzyme might be different from other DNMTs [27], its role in development of HCC may need further investigating.

Overall, the overexpression of DNMT mRNAs and high rate of p16 methylation were not only detected in the HBV-associated cancerous tissues, but also in HBV-associated cirrhotic tissues and even in histologically normal tissues. Moreover, there was a close correlation between DNMTs and p16 methylation. All the data together suggest that persistent HBV infection can stimulate the overexpression of DNMTs, particularly DNMT1, DNMT3A and DNMT3B, and induce hypermethylation of p16, resulting in the inactivation of p16 and indirect regulation of the progression of hepatocellular carcinogenesis.

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Disclosure of conflict of interest

The authors state that there are no conflicts of interest to disclose.

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