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LETTER TO THE EDITOR

The association of HBV infection with DNA methyltransferases expression in hepatocellular carcinoma

H.-P. LI[1], F.-M. YANG[2], B. GAO[3], Z. T. YU[3], J.-C. ZHANG[3*]

[1]GCP Office, [2]Department of Obstetrics and Gynecology and [3]Department of Laboratory Medicine, Taihe Hospital, Hubei University of Medicine, Shiyan 442000, Hubei, P. R. China

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Our previous research concluded that in addition to the high rate of p16 methylation found in 31/44 (70.5%) of hepatocellular carcinoma (HCC) patients (*1*), a much higher rate of p16 methylation was detected in the cancerous and cirrhotic tissues of HCC associated with hepatitis B virus (HBV) infection. However, the mechanism(s) of observed methylation changes following HBV infection are yet to be deduced. These observations were also reported by other groups (*2*). 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 (*3, 4*). 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. With respect to hepatocarcinogenesis, the overexpression of different DNMT proteins and mRNA have been reported (*5, 6*), but their relations with HBV infection status have not been analyzed. Thus, we hypothesized that HBV may promote the hypermethylation of p16, there by 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, tumor stage and differentiation. The relationship between the levels of DNMTs and p16 hypermethylation was also evaluated.

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 and the other group without HBV and/or HCV infection.

The detailed data of mRNA levels for DNMTs in groups with HBV infection and without it are shown in Table 1.

In our study, the average expression of DNMT1, DNMT3A and DNMT3B mRNA in cirrhotic and cancerous tissues with HBV infection was significantly higher than in tissues without it; 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. The average expression of DNMT2 mRNA in all the tissues and DNMT3B mRNA in HBV-associated non-cancerous tissues was found to be similar to the expression in non-HBV-linked tissues.

The average expression of individual DNMTs and their elevated mRNA level were significantly higher in HBV-associated tissues. The frequency of elevated mRNA expression

*Corresponding author. E-mail: fromzero1121@hotmail.com; phone: +867198801192.

Abbreviations: HBV = hepatitis B virus; HCC = hepatocellular carcinoma; DNMT = DNA methyltransferases

Table 1. mRNA expression of DNMT in cancerous, cirrhotic and non-cancerous tissues of HCC patients with different HBV infection status

Tissues	No.	DNMT1	DNMT2	DNMT3a	DNMT3b
Cancerous tissue	44				
With HBV infection	32	1.22±0.21*	0.75±0.14	1.35±0.23*	0.67±0.13**
HBsAg+,HBeAg+	7	1.35±0.26	0.86±0.12	1.39±0.22	0.72±0.14
HBsAg+,HBeAg-	18	1.23±0.23	0.75±0.13	1.34±0.20	0.68±0.13
Other markers+	7	1.10±0.17	0.68±0.11	1.30±0.18	0.57±0.11
Without HBV infection	12	0.85±0.24	0.66±0.11	0.94±0.16	0.50±0.08
Cirrhotic tissue	35				
With HBV infection	24	0.98±0.17*	0.64±0.13	0.99±0.19*	0.58±0.15**
HBsAg+,HBeAg+	7	1.00±0.21	0.69±0.15	1.05±0.20	0.63±0.16
HBsAg+,HBeAg-	10	0.96±0.17	0.65±0.13	0.96±0.18	0.58±0.14
Other markers+	7	0.91±0.17	0.62±0.12	0.90±0.18	0.54±0.13
Without HBV infection	11	0.70±0.14	0.60±0.14	0.72±0.16	0.39±0.12
Non-cancerous tissue	44				
With HBV infection	32	0.63±0.14**	0.55±0.12	0.66±0.23**	0.34±0.07
HBsAg+,HBeAg+	7	0.73±0.16	0.60±0.13	0.71±0.25	0.36±0.12
HBsAg+,HBeAg-	18	0.67±0.15	0.56±0.12	0.65±0.22	0.33±0.11
Other markers+	7	0.59±0.13	0.48±0.11	0.58±0.20	0.32±0.10
Without HBV infection	12	0.42±0.10	0.48±0.10	0.44±0.16	0.28±0.00

Note: (1) Cases with at least two positive antigens (HBsAg, HBeAg and HBcAg) in serum were included and patients with HCV infection were excluded from this study. (2) *P <0.05; **P <0.01.

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. The levels of DNMT1, DNMT3A and DNMT3B mRNAs in cirrhotic tissues were similar to those in cancerous tissues. Even in the non-cancerous tissues, an elevated expression of DNMT1 and DNMT3A was observed in the HBV infected group than in the uninfected group.

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.

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. HCC cell line HepG3B contains an integrated hepatitis B virus genome and can secrete HBsAg (*7*). HepG2 is a human hepatoblastoma cell line, with no evidence of a hepatitis B virus. 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 tumor cell lines than in normal cells, our study demonstrated that it is 3-fold higher in HepG3B than in HepG2 cells. This discrepancy may be caused by: (1) different research subjects or; (2) the role of HBV in Hep3B, where HBV stimulates the expression of DNMT3A.

In all cases with p16 hypermethylation present at least one kind of increased DNMT expression in cancerous tissues was present, 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 (*8*).

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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