行政院國家科學委員會補助專題研究計畫成果報告

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※ 潛伏性B型肝炎病毒感染在台灣地區慢性C型肝炎患者之臨床意義(II) ※

※ The Implications of Occult Hepatitis B Virus Infection in Patients with ※

※ Chronic Hepatitis C Virus Infection in Taiwan (II) ※

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一、中文摘要

在上一年度的報告中，吾人發現潛伏性 B 型肝炎病毒感染的確可以存在於 B 型肝炎表面抗原陰性的健康成人體內，而其盛行率在 C 型肝炎患者和一般成年人口則屬相當，約為 15%。在本年度的計畫，吾人繼續探討潛伏性 B 型肝炎病毒感染的分子病毒機轉。在 100 例 B 型肝炎表面抗原陰性的慢性 C 型肝炎患者中，利用 PCR 法偵測 HBV DNA 之存在，結果有 10 例在血清中可找 HBV DNA。為探討可能的基因體變異，吾人再以 PCR 和核酸定序法針對 pre-S、S、pre-core/core promoter 等基因作進一步之研究。結果顯示 1 例有 pre-S 基因缺損，2 例有 S 基因上 a 決定位之胺基酸變異。此外，吾人發現 8 例在 core promoter 上有新型的 T1802C/T1803G 雙核酸突變，其發生比例遠較 B 型肝炎表面抗原陽性之帶原者為高(80%比 10%，P<0.001)。吾人之研究結果顯示這個新發現的 core promoter 突變可能和這些病人體內無法測得 B 型肝炎表面抗原有關。

關鍵字：慢性 C 型肝炎、基因體變異、B 型肝炎病毒、潛伏性 B 型肝炎病毒感染

二、Abstract

Although occult hepatitis B virus (HBV) infections in individuals without detectable hepatitis B surface antigen (HBsAg) may occur and have been reported to be common in patients with chronic hepatitis C, the related molecular mechanisms remain unsettled. With polymerase chain reaction, serum HBV DNA was sought in 100 HBsAg-negative patients with chronic hepatitis C virus (HCV)-infection. In those with occult HBV infection, possible genomic variability of HBV was evaluated by amplification and direct sequencing of pre-S, surface and pre-core/core promoter genes. In total, 10 of the 100 patients (10%) had detectable serum HBV DNA, documenting an occult HBV infection. A deletion mutant in the pre-S gene was found in one patient and mutations of the *a* determinant of HBsAg were observed in 2. In addition, a novel core promoter mutant (a dinucleotide substitution: T-to-C at nucleotide 1802 and T-to-G at nucleotide 1803, T1802C/T1803G) was found frequently in patients with occult HBV infection as compared to sex- and age-matched HBsAg-positive patients (80% vs. 10%, $P < 0.001$). In conclusion, the data suggest occult HBV infection is not uncommon in chronic hepatitis C patients in Taiwan, and a novel core promoter mutant may be associated with the absence of circulating HBsAg in these patients.

Key words: chronic hepatitis C, core promoter, genomic variability, hepatitis B virus, occult HBV infection, pre-core gene, pre-S gene, surface gene.

三、Introduction

Hepatitis B virus (HBV) infection is usually diagnosed when circulating hepatitis B surface antigen (HbsAg) is detected [Kao and Chen 2000a]. However, a unique persistent infection known as "occult HBV infection", which is characterized by the prevalence of serum HBV DNA by using nested PCR assays has been identified in HbsAg-negative patients with or without serological markers of previous infection [Brechot et al., 1985; Liang et al., 1991; Zhang et al., 1993]. Several recent studies have indicated that this occult HBV infection is found commonly in patients with chronic HCV infection with variable frequency (50–87%) [Uchida et al., 1997; Zignego et al., 1997; Koike et al., 1998; Fukuda et al., 1999]. The high prevalence of the occult HBV infection in these patients has been suggested to be associated with more severe liver damage and even the development of hepatocellular carcinoma (HCC) [Sheu et al., 1992; Cacciola et al., 1999; Yotsuyanagi et al., 2000]. In addition, several studies suggested that occult HBV infection may correlate with a lack of response to interferon treatment in patients with chronic hepatitis C [Zignego et al., 1997; Cacciola et al., 1999; Fukuda et al., 1999]. Taken together, a low level HBV infection may contribute not only to the severity of HCV-related liver disease but also may be of prognostic importance.

Although previous studies have suggested that genetic variability of the HBV genome may be responsible for the lack of HbsAg [Yamamoto et al., 1994; Uchida et al., 1997; Fukuda et al., 1999; Cabrerizo et al., 2000; Schories et al., 2000; Weinberger et al., 2000], the mechanisms involved in the absence of circulating HbsAg in patients with occult HBV infection remain to be explored [Bréchot et al., 2001]. In this study, we investigated the association of viral genomic variability with occult HBV infection in Taiwan where HBV infection is hyperendemic.

四、Patients and Methods

Patients

Serum samples were studied retrospectively from 100 patients with chronic HCV infection (59 men, 41 women; mean age, 46±12 years). All of them were positive for both anti-HCV and HCV RNA, and were negative for HBsAg. Serum samples taken from each subject were stored at -70 ^{o}C until use. Occult HBV infection was defined as a positive serum HBV DNA by using the nested polymerase chain reaction (PCR) assay in an HBsAg-negative

individual with or without serologic markers of past infection.

Serological Markers

HBV serum markers (HBsAg, anti-HBs and anti-HBc) were sought using commercial enzyme immunoassays (Ausria II, Ausab and Corab, Abbott Laboratories, North Chicago, IL, USA). Anti-HCV was tested with a second-generation enzyme-linked immunoassay (Abbott Laboratories).

Detection of HCV RNA

Serum HCV RNA was assayed by reverse transcription (RT)-PCR with primers from the most conserved 5' untranslated region of the viral genome [Kao et al., 1998]. To avoid false-positive results, the methods described by Kwok and Higuchi to prevent cross contamination were applied [Kwok and Higuchi 1989].

Detection of HBV DNA

The presence of HBV DNA was assayed by two different PCR assays with primer pairs from the surface (S) and pre-core/core promoter genes of the viral genome (Table 1). Briefly, total DNA was extracted from 100 µl serum using QIAamp Blood kit (QIAGEN Ltd, Crawley, UK) and resuspended in 50 µl elution buffer. For the first stage PCR, a 25 µl of reaction mixture containing 2 µl of the DNA sample, 1x PCR buffer (10 mM tris-HCl pH 9.0, 50mM KCl, 1.5 mM MgCl2, 0.01% gelatin and 0.1% Triton X-100), 10 mM of each dNTP, 100 ng of each outer primer pair and 1 unit of Taq DNA polymerase was amplified in a thermal cycler (Perkin-Elmer Cetus, Norwalk, CT, USA) for 30 cycles. Each cycle entailed denaturation at 95 ^{o}C for 60 s, primer annealing at 55 ^{o}C for 30 s and extension at 72 ^{o}C for 60 s with a final extension step at 72°C for 7 min. After the first amplification, 1 µl of the PCR products was reamplified for another 30 cycles with 100 ng of each inner primer pair. The second round of PCR was done in the same manner as the first round. The amplified products were separated by electrophoresis in 3% agarose gel and stained by ethidium bromide. The sensitivity of our PCR assays reached 10 copies of HBV DNA per specimen by testing serial 10-fold dilutions of HBV DNA transcripts with known amounts (10^{8} copies/ml) as previously described [Kao et al., 2000b].

Amplification of pre-S gene and sequencing of surface and pre-core/core promoter genes

The complete pre-S gene was amplified further, and the pre-S deletion mutant was defined when a shorter PCR fragment was observed in addition to the expected-size product (564 bp) or only the shorter form was detected. For sequence analysis, nested PCR for amplification of surface and pre-core/core promoter genes was undertaken using respective

primer pairs (Table 1). Nucleotide sequences of the amplified products were directly determined by using fluorescence labelled primers with a 377 Sequencer (Applied Biosystems, Foster City, CA, USA). Sequencing conditions were specified in the protocol for the Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems). The inner primer pair was used as sequencing primers for both directions of each gene.

Statistical Analysis

Data were analyzed by Fisher's exact test where appropriate. A P value of less than 0.05 was considered statistically significant.

五、Results

Ten (10%) of 100 patients with chronic HCV infection were found with serum HBV DNA by both PCR assays, documenting an occult HBV infection (Table 2). Among them, 9 had antibody against hepatitis B core antigen (anti-HBc) and 7 had antibody against HBsAg (anti-HBs). To characterize the role of genomic variability of HBV in occult HBV infection, the pre-S gene was first amplified to detect the presence of pre-S deletion mutant and the nucleotide sequences of the S, pre-C, and core promoter genes of the predominant HBV strains were then determined in all of the 10 HBV DNA-positive chronic hepatitis C patients by using a direct sequencing method (Table 2). A deletion mutant in the pre-S gene of the HBV genome was found in only one patient (Case 9, Table 2). As to the putative amino acid analysis in the *a* epitope (amino acid 124 to 147) of the S gene, the results showed that most of the 10 HBV DNA-positive patients were infected with the wild type HBV isolates, and only 2 had amino acid changes in this region (Cases 9 and 10, Table 2). The sequences of the pre-C gene derived from the 10 HBV DNA-positive patients showed a pre-core stop codon mutant (codon 28 with a G-to-A change at nucleotide 1896) in 4 patients, and the presence of wild type strain was not necessarily associated with HBeAg positivity. Indeed, 6 patients who had a dominant population of wild type strain lacked HBeAg in their sera.

Nucleotide sequence analysis of the core promoter gene showed that none of the 10 patients had an 8-nucleotide deletion between nucleotide 1768 and 1775, and a dinucleotide exchange (A-to-T at nucleotide 1762 and G-to-A at nucleotide 1764, A1762T/G1764A) was observed in only one (Case 9, Table 4). Of interest, a novel core promoter mutant (a dinucleotide substitution: T-to-C at nucleotide 1802 and T-to-G at nucleotide 1803, T1802C/T1803G) was found in 8 of them (Cases 1 to 8, Table 4). To investigate the role of

this novel HBV core promoter mutant in the presence of low level HBV replication, the core promoter sequences were determined in additional 20 sex- and age-matched HBsAg-positive but HBeAg-negative patients and 2 (10%) of the controls had a core promoter T1802C/T1803G mutant. Thus the prevalence of core promoter T1802C/T1803G mutant in patients with occult HBV infection was significantly higher than that in those seropositive for HBsAg (80% vs. 10%, $P < 0.001$).

六、Discussion

HBV and HCV infections account for a substantial proportion of chronic liver disease including chronic hepatitis, cirrhosis and liver cancer. It is estimated that there are 350 million HBV carriers and 170 million HCV carriers worldwide [Kao and Chen 2000a]. HBV and HCV are transmitted parenterally and share common routes of infection, thus infection with both viruses may occur, particularly in areas where the two viruses are endemic and among people at high risk for parenteral infections [Chen et al., 1990; Pontisso et al., 1993]. Recently, several studies indicated that occult HBV infection can be found in patients with chronic HCV infection and has clinical implications in the pathogenesis of HCV-induced chronic liver disease [Sheu et al., 1992; Uchida et al., 1997; Zignego et al., 1997; Cacciola et al., 1999; Koike et al., 1998; Fukuda et al., 1999; Yotsuyanagi et al., 2000]. However, little is known about the mechanisms involved in the absence of circulating HBsAg in this special clinical setting [Bréchot et al., 2001].

Previous studies have suggested that point mutation, deletion or rearrangements in several genes of the HBV genome that interfere with gene expression or lead to the production of an antigenically modified S protein may be responsible for the lack of HBsAg [Yamamoto et al., 1994; Uchida et al., 1997; Fukuda et al., 1999; Cabrerizo et al., 2000; Schories et al., 2000; Weinberger et al., 2000]. For example, a single mutation at the common *a* determinant (amino acid 124-147) of HBsAg can cause a change in the immunological epitope [Yamamoto et al., 1994; Schories et al., 2000; Weinberger et al., 2000], and mutations or deletion in the pre-S gene can lead to the inhibition of the secretion of HBsAg [Cabrerizo et al., 2000]. In addition, an 8-nucleotide deletion between nucleotide 1768 and 1775 in the core promoter gene has been implicated to suppress the replication and expression of HBV DNA and thus result in the absence of serological markers despite the presence of HBV infection [Uchida et al., 1997; Fukuda et al., 1999]. However, the results from these previous

studies are not consistent and sometimes even conflicting. And thus, the association of hepatitis B viral genomic variability with the failure to detect serum HBsAg needs further investigation.

In the present study, it was intended to identify the regions of the viral genome that might be responsible for the lack of production of HBsAg. As shown in Table 2, a mixture of wild type virus and deletion mutant in the pre-S gene of the HBV genome was demonstrated in one patient (Case 9). This fact indicates that the HBsAg must be provided *in trans* by a wild type virus for the secretion of viral particles containing mutant genomes. In this sense, it is important to note that the deletion mutant is always accompanied by the wild type virus as seen in our case. The putative amino acid sequences were then analyzed in the *a* determinant of HBsAg, and the results showed that all but 2 of the patients were infected with the wild type HBV isolates (Cases 9 and 10, Table 2).

A pre-core stop codon mutant (codon 28 with a G-to-A change at nucleotide 1896) in 4 patients and a wild type strain in 6 who lacked HBeAg in their sera were found in this study. Similar situation has also been observed in healthy individuals before, and may be explained by the inefficient proliferation of HBV that contributes to the low level synthesis of HBeAg [Marusawa et al., 2000]. In addition, the poor immune attack would be responsible for the occurrence of few mutations in the pre-core region observed in these subjects with occult HBV infection. This condition might lead to persistent infection of wild type HBV despite the absence of serum HBeAg.

The nucleotide sequence analysis of the core promoter gene was also performed. The core promoter gene is located at the distal part of the X gene of HBV, which contains important elements for HBV replication including the liver specific enhancer II [Kramvis and Kew 1999]. Many cellular transcriptional factors also bind to these elements. Our data showed that none of the 10 patients had an 8-nucleotide deletion as was reported in Japan [Uchida et al., 1997; Koike et al., 1998; Fukuda et al., 1999], suggesting such a deletion is not associated with the negativity of serum HBsAg in the Taiwanese patients with occult HBV infection. In the meantime, a dinucleotide exchange (A-to-T at nucleotide 1762 and G-to-A at nucleotide 1764, A1762T/G1764A) was observed in only one (Case 9). This dinucleotide mutation is located within the nuclear protein-binding DNA sequences of the core promoter and enhancer II, thus this mutation may alter the transcriptional activity of HBV by changing the binding affinity of the nuclear proteins for the mutated sequences [Kramvis and Kew 1999]. Of interest, we found a novel core promoter mutant (a two-nucleotide substitution:

T-to-C at nucleotide 1802 and T-to-G at nucleotide 1803, T1802C/T1803G), and the prevalence of core promoter T1802C/T1803G mutant was significantly higher in patients with occult HBV infection than that in those positive for HBsAg (80% vs. 10%, $P < 0.001$). There are two highly conserved regions identified in the core promoter gene, nucleotides 1770 to 1808 and nucleotides 1813 to 1849, which are crucial in the regulation of transcription [Kramvis and Kew 1999]. Thus mutations within these regions may therefore affect the replication of the virus. However, further studies are needed to confirm these interesting and important findings.

In summary, it was found that occult HBV infection is not uncommon in patients with chronic hepatitis C in Taiwan where HBV infection is hyperendemic. In addition, a novel core promoter mutant may be associated with the absence of circulating HBsAg in these patients.

七、Acknowledgements

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Table 1. Sequences of primer pairs used for polymerase chain reaction to amplify pre-S/surface and pre-core/core promoter genes of hepatitis B virus genome

Primer no.*	Sequence (5'–>3')	Nucleotide position
Surface gene		
S-1s	AGAACATCGCATCAGGACTC	159 - 178
S-2a	CATAGGTATCTTGCGAAAGC	642 - 623
S-3s	AGGACCCCTGCTCGTGTTAC	181 - 200
S-4a	AGATGATGGGATGGGAATAC	619 – 600
Pre-core/core promoter gene		
PC1s	CAGACGGTCTGGAGCAAACC	1302 - 1321
PC2a	CAATGCTCAGGAGACTCTAAGGC	2043 - 2021
PC3s	CTCATCTGCCGGACCGTGTG	1562 - 1581
PC4a	GTCAGAAGGCAAAAAAGAGAG	1966 – 1946
Pre-S gene		
PS1s	GGGTCACCTTATTCTTGGGA	2814 - 2833
PS2a	CCCCGCCTGTAACACGAGCA	208 – 189
PS3s	TTGGGAACAAGATCTACAGC	2828 - 2847
PS4a	GTCCTGATGCGATGTTCTCC	176 - 157

s, sense; a, antisense.

Table 2. Genomic variability of hepatitis B virus (HBV) in chronic hepatitis C patients with occult HBV infection

Patient No.	Sex/ Age	Anti-HBs/ Anti-HBc	HBV variants					
			Pre-S deletion	S gene (aa 124-147)	Pre-core (codon 28)	Core promoter		
						8-nt deletion	A1762/G1764	T1802/T1803
1	M/41	–/+	–	wild	stop	–	wild	CG
2	F/51	+/+	–	wild	stop	–	wild	CG
3	M/67	+/+	–	wild	stop	–	wild	CG
4	F/57	+/+	–	wild	stop	–	wild	CG
5	F/55	–/+	–	wild	wild	–	wild	CG
6	F/43	+/+	–	wild	wild	–	wild	CG
7	M/60	+/+	–	wild	wild	–	wild	CG
8	M/49	+/–	–	wild	wild	–	wild	CG
9	M/51	+/+	+	S136L	wild	–	TA	wild
10	M/73	–/+	–	G145A	wild	–	wild	wild

Anti-HBs: antibodies against hepatitis B surface antigen; Anti-HBc: antibodies against hepatitis B core antigen; S: surface; aa: amino acid; nt: nucleotide; 8-nt deletion: an 8-nucleotide deletion between nucleotide 1768 and 1775; A1762/G1764: A at nucleotide 1762 and G at nucleotide 1764 (wild type strain); T1802/T1803: T at nucleotide 1802 and T at nucleotide 1803 (wild type strain); S136L: serine-to-leucine change at amino acid 136; G145A: glycine-to-alanine change at amino acid 145.