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C型肝炎患者之臨床意義 ※

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

※ Chronic Hepatitis C Virus Infection in Taiwan ※

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一、 中文摘要

儘管潛伏性B型肝炎病毒感染可在B型肝炎表面抗原陰性的病例中發現，但此種感染對慢性C型肝炎臨床病程和治療成效之影響仍有爭論，本研究計劃在於探討潛伏性HBV感染對本地C型肝炎患者之重要性。共收集210例HBsAg陰性之慢性C型肝病患者(110例慢性肝炎，50例肝硬化，50例肝癌)，利用PCR法偵測其體內有無HBV DNA存在。110例慢性肝炎患者均曾接受干擾素合併Ribavirin治療。另外100例HBsAg陰性之健康成年人作對照組。結果顯示14.8%之慢性C型肝病患者和15%之健康成年人為血清HBV DNA陽性，表示有潛伏性HBV感染。進一步分析後，發現HBV DNA陽性和慢性C型肝病之嚴重度與對合併療法之反應並無相關。因此，潛伏性HBV感染並不影響本地慢性C型肝炎患者之臨床病程，然而HBV病毒基因體突變和HBsAg陰性之相關性仍有待第二年度的研究。

關鍵詞：B型肝炎病毒、C型肝炎病毒、潛伏感染、慢性C型肝炎、合併療法

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 significance remains unsettled. With polymerase chain reaction, we searched for serum HBV DNA in 210 HBsAg-negative patients with hepatitis C virus (HCV)-related liver disease (110 with chronic hepatitis, 50 with cirrhosis, and 50 with hepatocellular carcinoma). Most of the patients had detectable antibodies to HBsAg (anti-HBs) or HBV core antigen (anti-HBc). All of the 110 chronic hepatitis C patients were treated with combination therapy of interferon plus ribavirin. In addition, 100 HBsAg-negative healthy adults served as controls. Thirty-one of the 210 patients (14.8%) had serum HBV DNA, as did 15 of the 100 healthy controls (15%). Serum HBV DNA was not detected in those negative for HBV serological markers. In patients with chronic HCV infection, the prevalence of occult HBV infection did not parallel with the severity of liver disease. In addition, the sustained response to combination therapy against hepatitis C was comparable between patients with and without occult HBV infection. In conclusion, occult HBV infection does not have clinical significance in patients with chronic hepatitis C. The association of viral genomic variability with the failure to detect HBsAg awaits further studies.

Key words: Hepatitis B virus, hepatitis C virus, occult infection, chronic hepatitis C, combination therapy.

三、 Introduction

Hepatitis B virus (HBV) and hepatitis C virus (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 [1]. 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 [2, 3]. The diagnosis of HBV infection is usually based on the detection of hepatitis B surface antigen (HBsAg), and the disappearance of this antigen indicates the clearance of HBV [1]. However, previous studies have shown that HBV DNA could be detected in patients with chronic liver disease who were negative for HBsAg but positive for antibodies to hepatitis B core antigens (anti-HBc) [4-6]. More recently, this so-called "occult HBV infection" has frequently been identified in patients with chronic HCV infection [7-9], and this occult infection may be associated with more severe liver damage and even the development of hepatocellular carcinoma (HCC) in such patients [10-12]. In addition, several studies have suggested that occult HBV infection may correlate with a lack of response to interferon treatment in patients with chronic hepatitis C [9, 12, 13]. 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. However, such association has been questioned and indeed needs further confirmation [14].

Taking advantage of the common HBV and HCV infections in Taiwan [2], we determined the prevalence of occult HBV infection in patients with HCV-related chronic liver disease and studied the possible influence of occult HBV infection on the clinical outcomes of the infected patients in the first-year study.

四、 Materials and Methods

Patients

Serum samples were retrospectively studied from 210 Taiwanese patients with histologically verified HCV-related chronic liver disease and 100 control subjects. These included (i) 100 healthy adults (52 men, 48 women; mean age, 40±7 years) with normal serum alanine aminotransferase (ALT) level and negative for both hepatitis B surface antigen (HBsAg) and antibodies against hepatitis C virus (anti-HCV). Among them, 82 were positive for both antibodies against HBsAg (anti-HBs) and hepatitis B core antigen (anti-HBc), 8 were positive for anti-HBc alone and 10 were negative for the three HBV serological markers of HBV infection; (ii) 50 patients (30 men, 20 women; mean age, 64±9 years) with hepatocellular carcinoma; (iii) 50 patients (28 men, 22 women; mean age, 57±10 years) with cirrhosis; and (iv) 110 patients (74 men, 36 women; mean age, 45±13 years) with chronic hepatitis C who had had received combination therapy of IFN alfa-2b (Intron A, Schering-Plough, Kenilworth, NJ, USA) 3 to 5 million units thrice weekly plus oral ribavirin (ICN Pharmaceuticals, Inc., Costa

Mesa, CA, USA) 1,200 mg daily for 24 weeks. The presence of HCV RNA and HBV DNA in the serum was determined before initiation of the combination therapy; at the end therapy; and 24 weeks after the therapy was discontinued. The response to combination therapy was classified into two patterns according to the serum ALT level and serum HCV RNA status. Patients who had normalized serum ALT levels (≤ 40 U/L) and undetectable serum HCV RNA at the end of therapy and during the follow-up period was considered to have a sustained response. Non-sustained response was defined as serum ALT levels that could not be normalized either at the end of therapy or during follow-up period without clearance of serum HCV RNA. Those with chronic HCV infection were positive for both anti-HCV and HCV RNA, and were negative for HBsAg. Of them, 181 (86%) were positive for anti-HBc and 127 (60%) were also positive for anti-HBs. To sum up, 20 (9.5%) of these patients with HCV-related chronic liver disease were negative for HBV serological markers and 190 (90.5%) were positive for anti-HBs and/or anti-HBc. The diagnosis of chronic liver disease was based on clinical and pathological grounds accepted generally including mild chronic hepatitis, moderate/severe chronic hepatitis, liver cirrhosis (LC) and HCC.

All the enrolled patients had no markers suggestive of autoimmune hepatitis including antinuclear antibodies, antimitochondrial antibodies and anti-smooth muscle antibodies. None had a history of alcoholism (> 50 gm/day), injection drug abuse, homosexuality, or hepatotoxic drug intake. Metabolic liver disease including hemochromatosis, Wilson's disease or α-1 anti-trypsin deficiency was excluded by clinical and laboratory data. Serum samples taken from each subject were stored at -70 ^{o}C until use.

Serological Markers

HBsAg, anti-HBs, anti-HBc and anti-HCV were tested with commercially available kits (Abbott Laboratories, North Chicago, IL, USA).

Detection of HCV RNA, Genotyping of HCV and Quantitation of HCV RNA

Serum HCV RNA was assayed by reverse transcription (RT)-polymerase chain reaction (PCR) with primers from the most conserved 5' untranslating region of the viral genome [15], and identification of HCV genotype by type-specific primers as previously described [16]. To avoid false-positive results, the methods described by Kwok and Higuchi to prevent cross contaminations were applied [17]. Serum HCV RNA level was also quantified by using a second generation bDNA signal amplification assay (Quantiplex-HCV, version 2.0; Chiron) with a detection limit of 0.2 MEq/mL [18].

Detection of HBV DNA

The presence of HBV DNA was assayed by 3 different PCR assays with primer pairs from the surface (S), core (C) and X 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 cDNA sample,

1x PCR buffer (10 mM tris-HCl pH 9.0, 50mM KCl, 1.5 mM $MgCl_2$, 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 °C for 60 s, primer annealing at 55 °C for 30 s and extension at 72 °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 [19]. Serum samples reactive for at least one of the 3 PCR assays were considered HBV DNA-positive.

Statistical Analysis

Data were analyzed by Chi-square test with Yates' correction or Student's t test where appropriate. A P value of less than 0.05 was considered statistically significant.

五、 Results

Of 210 patients with chronic HCV infection, 31 (14.8%) were positive for serum HBV DNA by different PCR assays, documenting an occult HBV infection. The prevalence of occult HBV infection in HCV carriers was comparable to that in healthy adults (15%, Table 2). In addition, none of the 10 healthy adults or 20 hepatitis C patients negative for all three HBV serological markers was positive for HBV DNA by our PCR assays.

In 100 healthy adults with various HBV serological markers, the prevalence of occult HBV infection ranged from 0 to 25% among subjects negative for all markers, those positive for anti-HBc alone and those positive for both anti-HBs and anti-HBc (Table 2). Although the prevalence was higher in those with anti-HBc alone than in those negative for all markers, the difference was not statistically significant.

In 210 patients with HCV-related chronic liver diseases, the prevalence of occult HBV infection did not parallel the severity of chronic liver disease (Table 2). It was highest in patients with HCC (22%) and was lowest in patients with cirrhosis (8%, P = 0.09). The mean age of HCC patients possessing HCV and occult HBV infection was similar to that of patients possessing HCV infection alone (60.1±8.5 vs. 60.1±7.9 years).

Among 110 patients with chronic hepatitis C, HCV genotypes 1b, 2a, 2b and mixed infection were found in 67, 29, 9, and 5, respectively (Table 3). When these patients were stratified by the presence or absence of occult HBV infection, there was no significant difference in clinicopathological features including gender distribution, mean age, percentage of transfusion history, mean peak serum ALT

level, histological severity, and distribution of HCV genotypes (Table 3). Although patients with HCV and occult HBV infection had a higher mean serum HCV RNA level than those with HCV infection alone, the difference was not statistically significant. In addition, the biochemical and virological responses to combination therapy of interferon and ribavirin did not differ between patients with and without occult HBV infection (Table 3). Overall, the sustained response rate was 38% and 39% in patients with serum HBV DNA and in those without serum HBV DNA, respectively..

Of the 16 patients with occult HBV coinfection before initiation of combination therapy for chronic hepatitis C, all lost serum HBV DNA at the end of therapy as determined by PCR assays, and 14 (88%) remained serum HBV DNA negative after the therapy was stopped for 6 months. No correlation was found between the sustained response and the loss of serum HBV DNA. On the contrary, a correlation was seen between sustained response and sustained loss of serum HCV RNA as anticipated (data not shown).

六、Discussion

HBV infection is diagnosed when circulating HBsAg is detected [1]. However, a unique persistent infection known as occult HBV infection which is characterized by the positivity of serum HBV DNA by using nested PCR assays has been identified in HBsAg-negative patients with or without serologic markers of previous infection (anti-HBs or anti-HBc) [4-6]. Several recent studies have indicated that this occult HBV infection can be found in patients with chronic HCV infection with variable frequency (50–87%) [7-13]. The high prevalence of the occult HBV infection in such patients has been suggested to have clinical implications in the pathogenesis of HCV-induced chronic liver disease. Nevertheless, by using highly sensitive PCR assays (Table 1), we found a comparable prevalence of occult HBV infection between patients with chronic HCV infection (14.8%) and healthy adults (15%), implying that the HCV carriers are not at increased risk for occult HBV infection than the general population in Taiwan. These findings are not unanticipated because most adults in Taiwan contracted HBV infection during their childhood, and superinfection of other viruses including HCV may occur thereafter [20]. In addition, the low level replication of HBV in occult HBV infection appears not to result from the interference by HCV [21] because the occult HBV infection is not necessarily accompanied with HCV infection as observed in the healthy controls.

The relationship between occult HBV infection and serological markers of HBV has been studied before, and the prevalence of occult HBV infection was usually higher in subjects positive for either or both anti-HBs and anti-HBc than in those negative for all serological markers (46-80% vs. 20-50%) [9, 12]. Although statistically not significant, our data also showed that healthy adults positive for either or both anti-HBs and anti-HBc had a higher serum HBV DNA positivity than those negative for all serological markers (16 to 25% vs. 0%, Table 2). The

possibility of persistent HBV infection in anti-HBc-positive individuals has been supported by recent studies showing that traces of HBV are often detectable in the blood for many years after clinical recovery from acute hepatitis despite the presence of serum antibodies against HBV and HBV-specific cytotoxic T lymphocytes [22, 23].

The clinical significance of occult HBV infection alone or in combination with HCV infection remains unsettled. Previous epidemiologic and molecular studies have indicated that persistent HBV infection may have a critical role in the development of HCC in HBsAg-negative patients [11, 12] and in woodchucks [24] that have once been infected by woodchuck hepatitis virus even after the apparent clearance of the virus. Recently, occult HBV infection has been shown to correlate significantly with the development of cirrhosis among HCV-infected patients [12]. Their data suggested that a masked HBV infection may interfere with the clinical outcome of chronic hepatitis C, and favor or accelerate the evolution to cirrhosis. Since cirrhosis is generally the most important risk factor for the development of HCC [1], occult HBV infection may thus favor neoplastic transformation in HCV-infected patients through its contribution to cirrhosis. In contrast, our results showed that the prevalence of occult HBV infection did not parallel the severity of chronic liver disease, and the mean age of HCC patients with HCV and occult HBV co-infection was comparable to that of patients with HCV infection alone. In addition, among patients with chronic hepatitis C the demographic, clinical, histological and virological features were comparable between those with and without occult HBV co-infection (Table 3). Taken together, our observations suggested that occult HBV infection may have little influence on the clinicopathological course of chronic HCV infection, at least in the Taiwanese patients. In addition, occult HBV infection has been claimed to promote HCV replication [7, 9]. However, we could not observe such association in our study (Table 3).

Occult HBV infection has been suggested to jeopardize the response to interferon therapy in patients with chronic hepatitis C [9, 12, 13]; however, its impact on the response to combination therapy remains unknown to date. Our results showed that the sustained response rate to combined interferon alfa and ribavirin therapy was similar between chronic hepatitis C patients with and without occult HBV infection (Table 3), and thus, low level HBV does not interfere the response to combination therapy against hepatitis C. In addition, the sustained virological response rate of HBV to the combination therapy remains unexplored. Our data showed that all of the 16 patients with HCV and occult HBV coinfection lost their serum HBV DNA at the end of the combination therapy, and 14 (88%) had a sustained virological remission after stopping therapy for 6 months. These results suggest that low level HBV is interferon/ribavirin-sensitive, and whether this combination therapy also works for the treatment of usual chronic active hepatitis B needs to be studied further.

In summary, we found that occult HBV infection is common in healthy adults seropositive for HBV markers as well as in patients with chronic hepatitis C in Taiwan where HBV infection is hyperendemic; nevertheless, the coinfection of low level HBV bears no effect on the clinicopathological status of chronic hepatitis C and the therapeutic response to combination therapy. The association of viral genomic variability with the failure to detect HBsAg will be investigated in the second-year study.

七、References

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Table 1. Sequences of primer pairs used for polymerase chain reaction to detect 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
Core gene		
C1s	CTGGGAGGAGTTGGGGGA	1730 - 1747
C2a	GTAGAAGAATAAAGCCC	2503 - 2487
C3s	GGTCTTTGTACTCGGAGGCTG	1763 - 1783
C4a	ATACTAACATTGACATTCCC	2455 - 2436
X gene		
X1s	CTAGCCGCTTGTTTTGCTCG	1282 - 1301
X2a	TTATGCCTACAGCCTCCTAG	1666 - 1647
X3s	GGTCTTACATAAGAGGACTC	1518 - 1537
X4a	GTTCACGGTGGTCTCCAT	1625 - 1608
Pre-S gene		
PS1s	GGGTCACCTTATTCTTGGGA	2814 - 2833
PS2a	CCCCGCCTGTAACACGAGCA	208 - 189
PS3s	TTGGGAACAAGATCTACAGC	2828 - 2847
PS4a	GTCCTGATGCGATGTTCTCC	176 - 157
Precore/core promoter gene		
PC1s	CAGACGGTCTGGAGCAAACC	1302 - 1321
PC2a	CAATGCTCAGGAGACTCTAAGGC	2043 - 2021
PC3s	CTCATCTGCCGGACCGTGTG	1562 - 1581
PC4a	GTCAGAAGGCAAAAAAGAGAG	1966 - 1946

s, sense; a, antisense.

Table 2. Prevalence of serum hepatitis B virus (HBV) DNA in 100 healthy controls and 210 hepatitis C carriers with different liver diseases

	No. Studied	HBV DNA	
		No. Positive	%
Controls	100	15	15.0
All HBV markers negative	10	0	0
Anti-HBc alone	8	2	25.0
Anti-HBc and anti-HBs	82	13	15.9
Hepatitis C infected patients	210	31	14.8
Chronic hepatitis	110	16	14.5
Liver cirrhosis	50	4	8.0*
Hepatocellular carcinoma	50	11	22.0*

HBV DNA detected by polymerase chain reaction with primer pairs from surface, core or X gene.

* $P = 0.09$.

Table 3. Demographic and clinical data of 110 chronic hepatitis C patients with and without serum hepatitis B virus (HBV) DNA

Characteristics	Serum HBV DNA*	
	Positive	Negative
No. of cases	16	94
Sex (M/F)	10/6	64/30
Age (yr)	47±16	45±11
Transfusion history	5 (31%)	24 (26%)
Peak ALT level (U/L)	151±83	124±78
Histology		
Mild chronic hepatitis	13 (81%)	66 (70%)
Moderate/severe chronic hepatitis	3 (19%)	28 (30%)
HCV genotype		
1b	11 (69%)	56 (60%)
2a	3 (19%)	26 (28%)
2b	1 (6%)	8 (8%)
Mixed	1 (6%)	4 (4%)
Serum HCV titer (MEq/mL)	2.1±4.2	1.6±3.8
Response to interferon plus ribavirin		
Sustained responder	6 (38%)	37 (39%)
Nonsustained responder	10 (62%)	57 (61%)

ALT, alanine aminotransferase; HBV, hepatitis B virus; HCV, hepatitis C virus.
* The difference between HBV DNA-positive and -negative groups in each item was not statistically significant.