附件一

行政院國家科學委員會補助專題研究計畫 ☑成果報告 □期中進度報告

B型肝炎病毒基因型及表面抗原T細胞抗原決定部位基因變異慢性B型肝炎病毒感染病程的影響3/3（計畫名稱）

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成果報告類型(依經費核定清單規定繳交)：□精簡報告 ☑完整報告

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中文摘要

[背景及目的]:擬探討B型肝炎病毒基因型對於兒童B型肝炎病毒慢性感染的臨床預後，尤其對於e抗原抗體轉變及肝細胞癌的影響。

[對象及方法]

以追蹤長達15年以上之460名B型肝炎帶原兒童以及26名B型肝炎相關之肝癌兒童為對象，研究帶原兒童初進入本研究時及追蹤最後，以及肝癌兒童在初診斷時之B型肝炎病毒基因型。B肝帶原兒童依其初進入本研究時的e抗原抗體狀況分為e抗原陽性組及e抗體陽性組。e抗原陽性組再根據追蹤過程是否失去e抗原而細分為e抗原(+/+)組，及e抗原(+/-)組。

[結果]

基因型B型之B肝病毒分別佔e抗原(+/+)組，e抗原(+/-)組，及e 抗體陽性組的73%，86%及76%。而基因型C型則分別佔其27%，8%及6%。基因型C型在e抗原(+/+)組較其他二組常見($p=0.01$)，其e抗原抗體血清轉變較基因型B型為緩慢($p<0.001$)。在長程追蹤過程中基因型轉換很少見(2.8%)，肝癌兒童亦以基因型B型為最常見。

[結論]

基因型B型之B肝病毒在台灣B肝病毒慢性感染兒童及肝癌兒童均為最主要之基因型。基因型C型感染者的e抗原抗體血清轉變較緩慢，可能造成成人期的嚴重肝疾病。

關鍵詞：B型肝炎，基因型，e抗原血清轉變，肝細胞癌

ABSTRACT

Background/Aim: To investigate the influence of hepatitis B virus (HBV) genotypes on the clinical outcome of chronic childhood HBV infection, especially on hepatitis B e antigen (HBeAg) seroconversion and the development of hepatocellular carcinoma (HCC). *Subjects and Methods:* 460 HBV carrier children followed-up for 15 years and 26 HBV-related HCC children were recruited. Genotyping of HBV was performed at enrollment and the latest follow-up of these 460 carrier children and at the diagnosis in HCC children. These subjects were divided into HBeAg (+) group and anti-HBe(+) group based on their HBeAg/anti-HBe status at enrollment. HBeAg(+) group was further divided into HBeAg (+/+) group and HBeAg (+/-) group depending on whether spontaneous HBeAg seroconversion occurred during follow-up. *Results:* Genotype B constituted 73%, 86%, and 76% in HBeAg (+/+), HBeAg (+/-) and anti-HBe(+) group, respectively. Genotype C was found in 27%, 8%, and 6% in HBeAg (+/+), HBeAg (+/-) and anti-HBe(+) group, respectively. Genotype C carriers were more prevalent in HBeAg (+/+) Group than the other two groups ($p=0.01$), and had a delayed HBeAg seroconversion compared with the genotype B carriers ($p<0.001$). Changes of genotype during follow-up were rare (2.8%). In those with HCC, genotype B was also the major type (74%). *Conclusions:* HBV Genotype B dominates in childhood chronic HBV infection and HCC in Taiwan. Genotype C delays HBeAg seroconversion in chronic HBV infection, and may imply a more severe liver disease in adulthood.

Key Words: hepatitis B, genotype, HBeAg seroconversion, hepatocellular carcinoma

INTRODUCTION

In hyperendemic areas, chronic hepatitis B virus (HBV) infection results mainly from infections contracted during infancy and early childhood.[1-3] Although complications of the chronic infection manifest mostly during adulthood, histologic changes of the liver begin early since childhood.[4] Study in children with chronic HBV infection can help understand the clinical course after primary HBV infection.

In the natural course of chronic HBV infection, seroconversion from hepatitis B e antigen (HBeAg) to antibody of HBeAg (anti-HBe) is important in implying that the host breaks the immune tolerance and enters into a low replication phase.[5] According to our previous longitudinal follow-up studies, the precore stop codon mutation,[6] core gene deletion,[7] and basal core promoter mutation may account for HBeAg seroconversion in children. Nevertheless, additional determinant factors still need to be examined.

HBV was first classified into genotypes A to D.[8] On the basis of a nucleotide divergence of greater than 8% of the whole genome, it is currently divided into eight genotypes, A to H.[9,10] Different HBV genotypes are associated with different mutations in the HBV precore and core promoter gene regions during HBeAg seroconversion.[11-13] Patients infected with genotype C have been shown to run a rapidly progressive course of liver diseases than those with genotype B infection.[14,15] In Asian people, compared to genotype B, genotype C is associated with a higher frequency of core promoter mutation, higher aminotransferase activities, and lower response rate to interferon therapy.[16] We have further documented that HBV genotype C is prevalent in HCC patients >50 years, while genotype B is prevalent in HCC patients without cirrhosis and <50 years of age.[17]

Studies for the clinical relevance of HBV genotypes were almost limited in adults only. Furthermore, most studies were cross-sectional, or were with a relatively short follow-up duration. One cross-sectional study briefly mentioned that the genotype of HBV has no effect on liver damage in children.[18] There were no long-term follow-up study in children and young adults in this regard. Longitudinal studies spanning decades would be more revealing[19,20] than cross-sectional studies because the extrinsic factors confounding the natural course of chronic HBV infection can be avoided.[21] We take advantage of our long-term follow-up of HBsAg carrier children,[4-7] and focused on the following aims in the present study: (1) To study the influence of HBV genotypes on the clinical course of chronic HBV infection in children, especially on HBeAg seroconversion; (2) To examine the possibility of superinfections by different genotypes of HBV or transition from one genotype to another during the long-term follow-up; (3) To investigate the genotype distribution pattern in childhood HCC.

SUBJECTS AND METHODS

Under parental consents, 460 HBsAg carrier children were followed every 6 months. They were enrolled from the outpatient clinic of the National Taiwan University Hospital in a prospective study starting around 23 years ago,[22] and a cross-sectional study regarding hepatitis B vaccination efficacy survey.[23] Each child was examined at 6-month intervals or more frequently if possible. A physical examination and blood test, mainly for HBV markers and blood biochemistry, were taken at each visit. These children were grouped according to the HBeAg/anti-HBe status at enrollment. The HBeAg (+) group was further divided into (1) HBeAg (+/+) group representing children with persistent HBeAg positivity during the follow-up; and (2) HBeAg (+/-) group representing those who underwent HBeAg seroconversion during follow-up. HBeAg seroconversion was defined as loss of serum HBeAg and emergence of positive anti-HBe on at least two consecutive follow-up visits. Anti-HBe (+) group consisted of children who were already HBeAg negative and anti-HBe positive at enrollment. The serum samples at enrollment and the latest one during follow-up were submitted for HBV genotype analysis. The HCC group consisted of 26 HBsAg-positive children. The diagnosis of all tumors was confirmed by histological examination except in one patient. The only patient without tissue proof was diagnosed by radiological finding and a serum α-fetoprotein level of 343,000 ng/ml. Their serum samples were assayed for HBV genotypes at the time of the diagnosis of HCC (Table 1). None of the children received interferon or lamivudine therapy when they were in the study.

HBV Markers (including HBsAg, anti-HBs, anti-HBc, HBeAg, anti-HBe) were assayed by radioimmunoassays using commercial kits (Abbott Laboratories, North Chicago, IL, USA). The level of alanine aminotransferase (ALT) was determined by an autoanalyzer (Hitachi 7450, Tokyo, Japan).

Determination of HBV Genotypes. The HBV genotypes were analyzed by using polymerase chain reaction (PCR) with type-specific primers.[24] If the results were not interpretable after a double check, we applied the restriction fragment length polymorphism (RFLP) method developed by Lindh et al.[25]

In brief, DNA was extracted from 20ul of serum by the SDS-proteinase K/phenol/chloroform method, and dissolved in 10 µl of RNase-free water. Two µl of the product was used for PCR in a reaction volume of 20µl containing 3.5 mM $MgCl_2$, 50 mM tris-HC1, 10 pmol each of the first pair of primers P1 and P2 covering nucleotide 2823 to 704 of the HBV genome. The condition of the first run PCR was described previously.[22] The product of the first PCR were subjected

for nested PCR using mix A (for identifying genotypes A, B, C) and mix B (for identifying genotypes D, E, F) with a universal sense primer and type-specific antisense primer, respectively. The procedure condition was the same as the first PCR. The nested PCR sensitivity was at 10^{-5} pg of HBV DNA.

The PCR-RFLP method adapted primers spanning from nucleotide position 256-796 as described by Lindh et al.[25] The PCR product was then subjected to the digestion of *Tsp*5091 and *Hinf*I respectively. After incubation with the enzymes, the PCR product was run at 3% agarose gel. The RFLP patterns were then compared with those published previously.[25] All experiments were done at least in duplicate to confirm the results.

Comparison of HBeAg seroconversion between Genotypes B and C. To determine the age difference of HBeAg seroconversion between genotypes B and C, we pooled the cases of HBeAg (+) group, and plot the curve of age vs. percentage of HBeAg seroconversion by their respective genotypes (Kaplan-Meier survival analysis). Anti-HBe (+) group was not included for the analysis because they had already undergone HBeAg seroconversion at enrollment. Acute exacerbation was defined as an abrupt increase in the ALT level up to > five folds of upper limit of normal (200 U/L in this study), but the other causes, such as drugs or other viruses, were excluded.[26]

Statistics. Chi-square test with Yates' correction, Mann-Whitney rank test, and Kaplan-Meier survival analysis were applied where appropriate. A p value of <0.05 is considered statistically significant.

RESULTS

Basic and biochemical data. The baseline and peak ALT levels of the three groups of chronic HBV-infected children and the HCC group were shown in Table 1. Their ages at enrollment and at diagnosis were different statistically, the children of HCC group was older than the HBV-infected groups (Mann-Whitney rank test, $p<0.001$). At the latest follow-up, the ages of three HBV carrier groups were about the same ($p>0.5$).

Genotype distribution. Genotype B is the predominant strain of HBV in these children, but the distribution was not even in the HBV chronic infection groups, it accounted for 73%, 86%, and 76% in HBeAg (+/+), HBeAg (+/-) and anti-HBe(+) groups, respectively, while genotype C accounted for 27%, 8%, and 6% in the three groups, respectively (Fig. 1). Genotype C was significantly more common in HBeAg (+/+) group than in HBeAg (+/-) and anti-HBe(+) groups ($p<0.001$). A total of 13 cihldren (2.8%) had changes in HBV genotypes in the 460 subjects during our long-term follow-up. They occurred in two sequential serum samples

(4% and 8% in HBeAg (+/-) and anti-HBe(+) groups, respectively). Also, a few occasions of a mixed infection with genotypes B and C were found in the same serum samples; 2% and 10% in HBeAg (+/-) and anti-HBe(+) groups, respectively. Genotype changes or mixed genotypes infections were not observed in HBeAg(+/+) group.

In the 26 children with HCC, serum samples in seven patients yielded no PCR product. Genotype B was found in 79% (15/19); genotype C in 11% (2/19), and mixed genotypes of B+C and genotypes A was found in one each of the HCC children (5%).

Comparisons between children with genotypes B and C. In order to study the possible effect of genotype on the rate of HBeAg seroconversion, all children with positive HBeAg at enrollment, i.e., HBeAg (+) group, were analyzed by their respective genotypes. Those with mixed genotypes or genotype changes were excluded in this analysis. The basic data between children with these two genotypes were comparable (Table 2). We plotted the cumulative HBeAg seropositive percentage vs. the ages of carrier children with these two genotypes and found genotype C had a delayed HBeAg seroconversion ($p<0.0001$, Fig. 2). To avoid the confounding effect of early HBeAg seroconversion caused by the elevated ALT levels at enrollment, we excluded 40 patients (30 in genotype B and 10 in genotype C) presented with elevated ALT (>60 U/L). We then analyzed the 340 children again for HBeAg seroconversion according to their follow-up duration. Genotype C still showed a delay of HBeAg seroconversion compared with genotype B (Kaplan-Meier survival analysis, median of follow-up duration when the seroconversion occurred: genotype C vs. genotype B=18.2 years vs. 10.2 years, $p=0.0001$).

DISCUSSION

This is the longest prospective study for HBV genotype (about 20 years) in the literature to date. This childhood study also supplements the lacking data of HBV genotype during the natural history of chronic HBV infection in the first two decades of life. Our results confirmed previous observation in adults that HBV genotype B is most common in Taiwan.[27] In the meantime, we also found that genotype C rather than genotype B can delay HBeAg seroconversion in the carrier children by two approaches: first, genotype C constituted a larger proportion in HBeAg (+/+) than the other groups; second, genotype C children seroconverted at an older age.

Genotype shifting during the follow-up period is a rare and interesting finding in the present study. Such a phenomenon was infrequently seen in adults and

occurred in acute exacerbation.[21] Though we did not specifically detect the superinfection or genotype changing during acute exacerbation in our prospective study, we showed that these incidents were exclusively found in carrier children after HBeAg seroconversion. This fact may imply that chances of repeated superinfection in childhood may occur in these children and resulted in genotype changes. The other possibility is that they might have mixed genotypes initially, with one genotype dominated the other. After HBeAg seroconversion, the major type was cleared by the host immune response and the minor type then dominated. Because either genotype B to C or genotype C to B was demonstrated, it seemed no advantage at the initial stage for either genotype of HBV.

Because severe HBV-related parenchymal liver disease is generally rare in children, we did not analyze the clinical outcome or histologic gradings of these HBsAg carrier children. Instead, we investigated the persistence of HBeAg in them. HBeAg seroconversion usually marks the subsidence of HBV replication and reflects the inactive disease status.[11,28] HBeAg (+/+) group consisted of children who did not undergo HBeAg seroconversion in the whole follow-up period up to a mean age of 20 years. That means their age of HBeAg seroconversion would be after 20 years at least (Table 1). Genotype C constituted a relatively larger proportion in HBeAg (+/+) group in comparison to HBeAg (+/-) and anti-HBe(+) groups ($p<0.001$). Thus, we speculate that those with genotype C infection may be more resistant to HBeAg seroconversion as is seen in HBV carrier adults.[29] The delayed HBeAg seroconversion may prolong the inflammatory process and consequently result in a more severe liver damage.[30] Accordingly, genotype C may be more virulent. Though peak ALT levels in genotypes B and C carriers were comparable in our study in children (Table 2), the possibility of severer liver disease occurring after adult life is likely.[31]

HCC is regarded to be the gravest complication of chronic HBV infection. In Taiwan, it had been shown that genotype B is the predominant type in patients with HCC younger than 35 years, while genotype C is more prevalent as the patients' age goes up.[17] Although the present study consistently demonstrated genotype B was also the most common genotype in childhood HCC, there existed differences from young adult HCC. First, most of childhood HCC (>80%) were accompanied by cirrhosis,[32] whereas the young adult HCCs infected by genotype B HBV were mostly non-cirrhotic.[17] Second, still a minority of childhood HCCs belonged to genotype C, while none of the young adult HCC was genotype C. Thus, the carcinogenesis of childhood HCC may be not exactly the same as that in adult HCC. Further study is mandatory to address this question.

In conclusion, HBV genotype B accounts for >70% of chronic HBV infection

in Taiwan, and HBeAg seroconversion is delayed in those infected with HBV genotype C. Changing and mixing genotypes occur only in those who had undergone HBeAg seroconversion in childhood HBV chronic infection. For childhood HCC, genotype B is still the dominant one, and the proportion approximates that in HBsAg carrier children.

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Table 1. The Biochemical Data in Children with Chronic HBV Infection and HCC

	M:F	Peak ALT (U/L) mean±SD (range)	Age at enrollment (mean±SD)*	Age at latest follow-up (mean±SD)
HBeAg (+) Group				
HBeAg (+/+)	97:63	42±102 (1-878)	5.6±4.4	20.1±5.2
HBeAg (+/-)	139:99	63±65 (2-663)	6.9±4.4	22.3±4.9
Anti-HBe(+) Group	43:19	26±45 (3-308)	8.1±4.4	24.6±5.3
HCC Group	18:8	48±36 (10-185)	10.9±4.3	

*: The ages at enrollment are statistically different among the four groups (Mann-Whitney rank test, $p<0.001$).

Table 2. Comparisons of Patients with Genotypes B and C in HBeAg(+) Group

	Genotype B (n=320)	Genotype C (n=60)
Sex (M:F)	187:133	38:22
Age at enrollment (yr)	6.3±4.3	6.2±4.2
Follow-up duration (yr)	14.8±4.6	14.7±4.0
Peak ALT, median (U/L)	172±221, 81	159±289, 40
Acute exacerbation (%)	25	15
HBeAg seroconversion[a] (%)	64	33

[a]$p<0.0001$ by Chi square test with Yates' correction, the differences in the other parameters were not statistically significant. Those with mixed genotype and genotype changes were excluded.

FIGURE LEGENDS

Figure 1. Genotype distribution in the three groups of chronic HBV-infected children and children with HCC. HBeAg (+/+) group: persistent HBeAg seropositive; HBeAg (+/-) group: underwent HBeAg seroconversion during follow-up; anti-HBe(+) group: already anti-HBe positive at enrollment) and HCC children. Serum HBV DNA could not be detected by PCR in seven HCC children.

Figure 2. Children positive for HBeAg were analyzed according to their HBV genotypes. Those with genotype C had a significant delay of HBeAg seroconversion as compared with genotype B in terms of their age of seroconversion (Kaplan-Meier survival curve, $p<0.0001$). Cases with genotype changes and mixed genotypes were excluded from the analysis.