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兒童慢性B型肝炎病毒感染自然病程影響因子之長程研究：病毒量及病毒全長基因變化之探討(3/3)
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中文摘要

第一部份：慢性 B 型肝炎病毒感染兒童在 e 抗原抗體轉變自然史中之病毒量變化。目的背景：本研究探討慢性 B 型肝炎病毒(HBV)感染兒童在 e 抗原抗體轉變自然史中之血清病毒量變化。方法：58 名慢性 B 型肝炎病毒感染兒童合乎下列標準：進入本研究時 ALT 值正常，追蹤 10 年以上，無抗病毒藥物治療，追蹤期間 e 抗原抗體自然轉變。依據其 e 抗原抗體轉變後之 HBV DNA 濃度將之分爲二組：(1)低病毒血症組：一過性或者從未有>10^4 個病毒/ml(35 人)，(2)浮動性高病毒血症組：至少有兩次相隔一年以上>10^4 個病毒/ml(23 人)。腹部超音波，ALT 值及 HBV DNA 值每年至少偵測一次。另有 14 名未發生 e 抗原抗體轉變者作爲對照組。HBV 核前區(nt1896)基因及基因型亦被測定。結果：58 名研究組兒童最初 HBV DNA 爲 $10^{8.4\pm1.0}$ 個/ml，最後降至 $10^{2.9\pm2.0}$ 個/ml。他們進入本研究時，最高 HBV DNA 值時，最高 ALT 值時，e 抗原抗體轉變時，及最後的年齡分別爲 7.0±3.7，13.4±5.8，16.3±6.0，17.2±5.8，及 23.7±4.1。HBV 核前區變異在浮動高病毒血症組高於低病毒血症組(45.5%對 7.1%，P=0.006)。B 型肝炎基因型對於病毒量無明顯影響。在 e 抗原抗體轉換後，平均最高 ALT 值爲 26U/L，無人呈現持續 ALT 值續 ALT 值上昇現象。結論：一般而言，這些年輕時發生 e 抗原抗體轉變者病毒量會下降，ALT 值正常，轉變病程平順。未來更長程的追蹤研究將有助於了解在兒童及青少年期 e 抗原抗體轉變的意義。

第二部份：慢性 B 型肝炎病毒感染及肝癌兒童所分離出之 B 型肝炎病毒全長基因之研究。目的背景：過去對於長程追蹤之慢性 B 型肝炎病毒感染兒童及兒童肝癌患者 B 型肝炎病毒的全長基因變化並不瞭解。方法：21 名病程不同之 B 肝病毒慢性感染兒童及 7 名肝癌兒童進入本研究。以 PCR 及定序方法測定其 B 肝病毒全長基因序列。以建立基因樹的方法，我們分析 B 肝病毒序列在上述二組兒童之異同，並與國際文獻所報告的亞洲肝癌成人 B 肝病毒序列作比較。結果：基因樹分析發現肝癌兒童與慢性 B 型肝炎病童之 B 肝病毒序列分別聚集在不同的羣落。並在表面抗原前二基因(Pre-S2)12 個核苷酸及其他多個特定區發現肝癌兒童特有的 B 肝病毒序列刪除的現象。結論：多數肝癌兒童的 B 肝病毒基因相當特別，含有核前二區基因刪除等特別的序列，並與慢性 B 肝帶原兒童的序列不同，值得注意。

關鍵詞：B 型肝炎病毒(HBV)，HBV DNA，B 型肝炎 e 抗原抗體轉變，全長序列，種系演化樹，慢性 B 肝病毒感染，肝細胞癌

ABSTRACT

PART I : **Viremia Profiles in Children with Chronic Hepatitis B Virus Infection and Spontaneous e Antigen Seroconversion**

Aim/Background: This study investigated the viremia profiles in children with chronic hepatitis B virus (HBV) infection and spontaneous hepatitis B e antigen (HBeAg) seroconversion. **Methods:** 58 children with chronic HBV infection met the following criteria: normal alanine aminotransferase (ALT) at enrollment, followed for >10 years, no antiviral treatment, and having undergone spontaneous HBeAg seroconversion during follow-up. They were grouped according to the post-HBeAg seroconversion HBV DNA levels: (i) low viremia: transient or never $\geq 10^4$ copies/ml (n=35) (ii) fluctuating high viremia: $\geq 10^4$ copies/ml detected at least twice at intervals >1 year (n=23). Abdominal sonography, ALT, and HBV DNA levels were assessed annually. Another 14 non-seroconverted children served as controls. The precore mutant (nt1896) and genotypes were examined. **Results**: The initial HBV DNA level of the 58 seroconverters was $10^{8.4\pm1.0}$ copies/ml and decreased to $10^{2.9\pm2.0}$ copies/ml at the end of follow-up. Their mean ages at enrollment, peak HBV DNA, peak ALT, HBeAg seroconversion, and final follow-up were 7.0±3.7, 13.4±5.8, 16.3±6.0, 17.2±5.8, and 23.7±4.1 years, respectively. The precore mutant appeared more often in the fluctuating high viremia group than in the low viremia group (45.5% vs. 7.1%, p=0.006). HBV genotypes had no effect on the viremia profiles. After HBeAg seroconversion, the median peak ALT was 26 U/L and none had persistent abnormal ALT levels.

Conclusions: Generally, these young seroconverters had decreased viral loads, normal ALT levels, and uneventful courses after HBeAg seroconversion during our follow-up. A longer follow-up is necessary to elucidate the significance of HBeAg seroconversion occurring in childhood and young adulthood.

Part II. Full-Length Hepatitis B virus genomes isolated from children with chronic hepatitis B virus infection and hepatocellular carcinoma

Aims & Background: The changes of the whole HBV genome associated with different courses of childhood chronic hepatitis B (HBV) infection and HBV-related hepatocellular carcinoma (HCC) remain unclear and were under investigation in this study. **Methods:** We collected the serum samples from 21 children with different clinical courses of chronic HBV infection and from 7 children with HCC. The full-length HBV genomic sequences were obtained by PCR and sequencing method. Using phylogenetic tree construction, we analyzed the difference between HBV genomes from childhood HCC and those from other two groups of patients with either childhood chronic HBV infection or adult HCC. **Results:** Phylogenetic

analysis indicated that the majority of HBV genomes from children with HCC clustered in a specific class that was distinguishable from HBV genomes of either chronic HBV infection or adult HCC. Sequence analysis identified a consensus 12-bp pre-S2 deletion and other characteristic mutations throughout the genome. **Conclusion:** The majority of HBV genomes isolated from children with HCC contain unique mutations, such as a short pre-S2 deletion, that may be associated with early HCC development.

Key words: hepatitis B virus (HBV), HBV DNA, hepatitis B e antigen seroconversion, full length sequence, phylogenetic tree, chronic hepatitis B vidrus infection, hepatocellular carcinoma

PART I. VIREMIC PROFILES IN CHILDREN WITH CHRONIC HEPATIITS B INFECTION AND SPONTANEOUS e ANTIGEN SEROCONVERSION

INTRODUCTION

Chronic hepatitis B virus (HBV) infection is a health problem worldwide.1 In hyperendemic areas such as Taiwan, most chronic HBV infection begins in infancy and early childhood.2, 3 It usually leads to a chronic infection,4 which may result in serious complications in adult life.5,6 After HBV acquisition in infancy or childhood, the virus replicates actively in the initial years, which is an immune-tolerant stage with high HBV DNA levels and normal alanine aminotransferase (ALT) levels in most cases. Whereas adults generally show a gradual loss of tolerance to HBV and enter an immune-clearance stage, 7 there are few viremia profile reports in children. The host-virus interaction leads to acute exacerbation and subsequent hepatitis B e antigen (HBeAg) seroconversion. Previously, we observed the mutations of the precore gene,8 basal core promoter,9 and core gene deletion10 in the process of HBeAg
seroconversion in children.

Current treatment modalities for chronic hepatitis B seek to achieve HBeAg seroconversion, normal ALT, and a sustained suppression of the HBV DNA replication.11 These goals can occur spontaneously or achieved with antiviral therapy. Some adult HBeAg seroconverters would experience rebound viral replication, and are generally regarded as difficult to manage.12 This study was aimed to investigate whether HBeAg seroconversion in childhood, adolescence, or young adulthood might
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lower the HBV replication early and lead to a relatively benign course of chronic HBV infection.

Most of the previous studies have several pitfalls: (i) the blood sampling was done mostly in a cross-sectional manner when ALT levels were elevated, which often reflects the results of the virus-host interaction; (ii) the study subjects were a biased diseased population, not the community-based population; and (iii) previous pediatric reports did not use real-time polymerase chain reaction (PCR), the most sensitive method to measure the viral load. A prospective cohort study recruiting a large community-based population starting from the initial phase of HBV infection, perhaps in early childhood, would be more revealing. Such a prospective study would allow us to observe the natural history and monitor the disease outcome in children, adolescents, and young adults who undergo spontaneous HBeAg seroconversion.

SUBJECTS AND METHODS

Children with chronic HBV infection were recruited from a community-based general population through (i) four cross-sectional seroepidemiological surveys of HBV markers conducted in 1984, 1989, 1994, and 1999,13 (ii) a prospective screening program for children of HBsAg seropositive mothers, and (iii) the outpatient clinic of the National Taiwan University Hospital as part of a prospective study that began 25 years ago. A physical examination, blood tests for ALT, HBV seromarkers (including HBsAg, anti-HBs, anti-HBc, HBeAg, anti-HBe), α-fetoprotein, and abdominal sonography were done at each visit at 6-month intervals.

Of these children with chronic HBV infection, 72 became the subjects of this study. Of these, 58 met the following criteria and were enrolled in this study: (i) HBeAg positive and normal ALT at enrollment, (ii) age at enrollment <15 years, (iii) follow-up duration >10 years, (iv) no antiviral treatment given, and (v) underwent spontaneous HBeAg seroconversion to anti-HBe during follow-up. The other 14 patients served as the control group. They met the same criteria except the last: they were persistently HBeAg positive and did not undergo HBeAg seroconversion. The sex ratio and follow-up duration of the control group were similar to those of the study group (Table 2). HBV DNA was quantified using real- time PCR at least once each year. The study protocol was approved by the Institutional Review Board of the National Taiwan University Hospital, and the patients themselves or their guardians signed the informed consent to collect serum samples.

We subdivided the 58 seroconverters into two groups based on their post-seroconversion serum HBV DNA levels: (1) the low viremia group had viral loads of persistently <104 copies/ml (n=35) and (2) the fluctuating high viremia group had occasional episodes of viremia >104 copies/ml more than 6 months after HBeAg seroconversion (n=23). We used 104 copies/ml as the cut-off because this is the current indication for antiviral treatment for HBeAg-negative hepatitis patients.14 To ensure that the high viremia episodes were not insignificant, transient phenomena, we defined the fluctuating high viremia group as having two or more episodes of >104 copies/ml in an interval of more than 1 year.

HBV serological markers. The HBV seromarkers were measured using enzyme immunoassays (EIA, Abbott Laboratories, North Chicago, IL, USA). ALT levels were determined by an autoanalyzer (Hitachi 7450, Tokyo, Japan).

HBV DNA quantification using real-time PCR. The detailed nucleotide sequences and PCR procedures were described previously.15, 16 Briefly, HBV DNA was extracted from 50 ul of serum and the nucleic acids were re-dissolved in 50 µl of H2O and subjected to PCR. The primers cover HBV nucleotide positions 1261-1279 and

1600-1580. The anchor probe is at nucleotide positions 1552-1576 and the sensor probe is at nucleotide positions 1533-1550 of HBV DNA. The real-time PCR measurement was performed by using LightCycler analysis software 3.5 (Roche Diagnostics Applied Science, Mannheim, Germany). The linear range of HBV DNA was 10^2-10^{11} copies/ml and the sensitivity of this method was $5x10^2$ copies/ml of HBV in serum.

HBV genotyping. The latest serum samples of the 58 seroconverters were analyzed. Briefly, the HBV genotypes were analyzed by using PCR with type-specific primers. The procedures were described previously.

Detection of the precore 1896 stop codon mutant. The latest serum samples of the 58 seroconverters were used to detect the precore 1896 stop codon mutant. Nested PCR was performed using two pairs of primers covering the HBV precore and core regions. The procedures were described previously.

Statistics. To compare the low viremia, fluctuating high viremia, and nonseroconverter groups, the following statistical methods were used. The Chi-square test with Yates' correction was used to analyze the variables gender and precore mutant. Fisher's exact test was used to analyze the genotype. The Kruskal-Wallis test was used to compare the peak ALT levels with ALT levels expressed as the median and range. HBV DNA levels were log transformed and subjected to Student's *t* test. A $p < 0.05$ was considered statistically significant. The data were expressed as the mean $\pm$ standard deviation.

RESULTS

The general profiles of the serum HBV DNA of the 58 HBeAg seroconverter children are described in Table 1. The majority (77.6%, 45/58) of these patients were thought to have acquired the HBV infection from their mothers because their mothers were HBsAg positive. They initially had a high serum HBV DNA levels (108.4±1.0 copies/ml), which rose further to peak levels of 109.1±0.9 copies/ml, and then decreased to 102.9±2.0 copies/ml at the end of follow-up. Generally, the seroconverters had a peak HBV DNA level at the mean age of 13.4 years, followed by a peak ALT level at 16.3 years, and then HBeAg seroconversion occurred at 17.2 years of age (Table 1 and Fig. 1). There were exceptions: (1) the peak ALT occurred before the peak HBV DNA in seven cases and the interval between these two events ranged from 0.2 to 6.8 years; (2) the peak HBV DNA occurred 6.6 and 1.0 years after HBeAg seroconversion in two cases; and (3) the peak ALT occurred after HBeAg

seroconversion in seven cases at intervals ranging from 0.2 to 15.1 years. No children underwent HBsAg seroconversion at the final follow-up. The mean age at enrollment and the age at the final follow-up of the non-seroconverted children in the control group were 8.7±4.5 and 25.2±3.5 years, respectively. Their peak ALT and highest and lowest HBV DNA levels did not differ from those of the seroconverters in the pre-HBeAg seroconversion phase (Table 2). All of the children are still being followed and none of them show any signs of decompensated liver diseases, or HCC on physical examination, blood tests, and abdominal ultrasound.

HBV DNA and ALT profiles before and after HBeAg seroconversion. HBV DNA and ALT profiles before and after HBeAg seroconversion of these 58 seroconverter children are described in Table 2.

Post-HBeAg seroconversion low viremia group. A transient viremia of $>10^4$ copies/ml was found in 21 of the 35 cases (60%) in the low viremia group. Such events occurred an average of 2.0±1.5 years after HBeAg seroconversion.

Post-HBeAg seroconversion fluctuating high viremia group. In the post-HBeAg seroconversion phase, the first up-surge viremia of $>10^4$ copies/ml occurred 2.1±2.2 years after HBeAg seroconversion; the second episode of high viremia came 4.1±2.8 years after HBeAg seroconversion. There were 3.3±1.3 episodes of high viremia during the 9.2±5.5 year follow-up period after HBeAg seroconversion in the fluctuating high viremia group. All of the viremia episodes fluctuated above 10^4 copies/ml, but never persisted for longer than 1 year.

Precore mutant. This mutant was detected in 38% of the seroconverters (22/58). Fourteen seroconverters with the precore mutant were in the fluctuating high viremia group (14/23, 60.9%), which was much higher than the rate in the low viremia group (8/35, 23%; Table 3). Due to the longer follow-up duration after HBeAg seroconversion in the former group, there was concern that the emergence of the precore mutant simply reflected the natural history, i.e., the longer the duration of infection after seroconversion, the more likely the precore mutant is to occur. A multiple logistic regression analysis was performed, and the precore mutation was found to be significantly associated with the fluctuating high viremia vs. the low viremia group (odds ratio=4.4, 95% confidence interval =1.3-14.3, p=0.016), but not the follow-up duration after HBeAg seroconversion ≥ 9 years vs. <9 years (odds ratio=2.3, 95% confidence interval=0.56-9.09, *p*=0.25). Nine years is the mean follow-up duration for the fluctuating high viremia group, and was used as the

cut-off. The viremia profile, rather than a longer follow-up duration, was the significant independent factor associated with the precore mutation.

In the fluctuating high viremia group, there were six cases with an abnormal ALT level after HBeAg seroconversion and four of them had the precore mutant (see below). Of the remaining 17 cases with a normal ALT in this group, 10 had the precore mutant. The prevalence of the precore mutant did not differ between those with normal and abnormal ALT levels in the fluctuating high viremia group (p=0.73, Fisher's exact test with Yates' correction). In the low viremia group, four of the six cases with an abnormal ALT had the precore mutant. Of the 29 cases with a normal ALT level in the low viremia group, only four cases had the precore mutant. The precore mutant was more prevalent in those with an abnormal ALT (Fisher's exact test with Yates' correction, p=0.005).

Abnormal ALT in fluctuating high viremia group after HBeAg seroconversion. Six patients had these characteristics. In the four that had precore mutants, their ALT continued to fluctuate, but never exceeded 80 U/L after HBeAg seroconversion. Their peak HBV DNA levels were 104.6, 106.7, 105.7 and 107.4 copies/ml, respectively. The remaining two patients lacked the precore mutant and had mild ALT elevations after HBeAg seroconversion of 54 and 56 U/L, respectively. Their peak HBV DNA levels were 104.6 and 104.3 copies/ml, respectively.

Maternal HBsAg. Forty-five mothers of the 58 seroconverters were HBsAg positive and the children were thus presumed to have acquired the HBV infection perinatally. There was no difference between the children infected perinatally or horizontally in terms of the age of HBeAg seroconversion (16.8±5.5 vs. 19.0±6.8 years, p=0.22). However, none of the horizontally infected children (0/13) had an ALT flare-up after HBeAg seroconversion as compared with nine of the perinatally transmitted children who had an abnormal ALT after HBeAg seroconversion (9/45; *p*=0.08, Fisher's exact test).

DISCUSSION

This prospective long-term follow-up study illustrated the natural course of serum HBV DNA levels and their relationship to ALT levels and HBeAg seroconversion in children and young adults. A high HBV DNA viral load is found universally in the immune-tolerant phase in children with chronic HBV infection. The immune-clearance phase follows, during which ALT flare-ups occurred frequently and the peak ALT usually occurred after the peak HBV DNA.18We determined the average intervals between the successive peak HBV DNA levels, peak ALT levels,

and HBeAg seroconversion in children and adolescents in the natural history of chronic HBV infection (Fig. 1).

The current goals of antiviral treatments are to achieve sustained low viral replication after HBeAg seroconversion. How many subjects with spontaneous HBeAg seroconversion in childhood, adolescence, or young adulthood eventually develop a flare-up viremia (>104 copies/ml) and become HBeAg negative chronic hepatitis B victims?We found that 40% (23/58) of the seroconverters had more than one flare-up of HBV DNA levels and 20% (12/58) of them had ALT flare-ups after spontaneous HBeAg seroconversion. Nevertheless, all the peak ALT levels were less than two times the upper limit of normal. Therefore, none of these young seroconverters, who might experience both persistent high viremia episodes and an occasional ALT flare-up, met the current indications for antiviral treatment as a case of HBeAg-negative chronic hepatitis. We need to continue monitoring these patients to see if they develop persistent liver damage later in life.

The precore 1896 stop codon mutant is an important factor for persistent viral replication after HBeAg seroconversion, and may prevail in one-half to two-thirds of HBeAg-negative children and adults with chronic HBV infection in Taiwan. 8,9,19An abnormal ALT after HBeAg seroconversion is associated with the emergence of the precore mutant in adults.20 As expected, this mutant appeared more frequently (60.9%) in the fluctuating high viremia group than in the low viremia group (22.9%). We recognized its association with an abnormal ALT level in the low viremia group but not in fluctuating high viremia group. It is likely that fluctuating high viremia itself is important enough in inducing an abnormal ALT and minimized the role of the precore mutant. Continuous monitoring of such patients, including the ALT and viremia profiles, can help to elucidate the role of the precore mutant in the post-HBeAg seroconversion phase.

In conclusion, children with chronic HBV infection carry a high viral load in the immune-tolerant phase, and it decreases dramatically after they undergo HBeAg seroconversion. Forty percent of these spontaneous seroconverters still had fluctuating high viremia (>104 copies/ml) after HBeAg seroconversion. The precore 1896 mutant after HBeAg seroconversion is more prominent in fluctuating high viremia group. None of them warranted treatment after HBeAg seroconversion. The outcome of these young HBeAg seroconverters appeared to be relatively benign during our follow-up, however, continuous follow-up is necessary to make a final conclusion.

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Table 1. HBV DNA and ALT Profiles Before and After HBeAg Seroconversion in Nonseroconverters and Seroconverters

		Before seroconversion	After seroconversion
Follow-up period, y	16.6 ± 2.8	10.3 ± 4.9	6.5 ± 5.0
Median peak ALT level, U/L (range)[a]	159 (80–1166)	277 (10–1254)	26 (14–164)
Peak HBV-DNA level, $\log_{10}$ copies/mL[a]	9.2 ± .5	9.0 ± .9	5.0 ± 1.7
Lowest HBV-DNA level, $\log_{10}$ copies/mL[b]	7.0 ± 1.6	6.4 ± 1.7	—
$<10^2$ copies/mL, % (no.)	0	1.7% (1)	34.5% (20)
10^2–10^4 copies/mL, % (no.)	7.1% (1)	13.8% (8)	60.3% (35)
$>10^4$ copies/mL, % (no.)	92.9% (13)	84.5% (49)	5.2% (3)

NOTE. There were 9 male and 5 female nonseroconverters and there were 35 male and 23 female seroconverters. There were 12 patients with genotype B and 2 patients with genotype C among the nonseroconverters and 53 genotype B and 5 genotype C among the seroconverters.

[a]No difference between nonseroconverters and seroconverters in pre-HBeAg phase (Kruskal–Wallis test, $P = .27$, .52, and .29 for ALT level, peak HBV-DNA level, and lowest HBV-DNA level, respectively).

[b]No data available for the mean lowest HBV-DNA level after HBeAg seroconversion because 20 of the children had less than 10^2 copies/mL, which is below the detection level.

Table 2. The Time Course Of Viremia Profiles Of 58 Children With Chronic Hbv Infection And Spontaneous Hbeag Seroconversion

Maternal HBsAg status (positive:negative)	45:13
Age at enrollment, y	7.0 ± 3.7
Age at peak HBV-DNA level, y	13.4 ± 5.8
Age at peak ALT level, y	16.3 ± 6.0
Age at HBeAg seroconversion, y	17.2 ± 5.8
Age at HBeAg seroconversion in those with positive maternal HBsAg, y	16.8 ± 5.5
Age at HBeAg seroconversion in those with negative maternal HBsAg, y[a]	19.0 ± 6.8
Age at final follow-up evaluation, y	23.7 ± 4.1
Interval between the ages at peak ALT level and HBeAg seroconversion, y[b]	1.0 ± 4.2
Interval between the ages at peak HBV-DNA and ALT level, y[c]	2.9 ± 4.2
Interval between the ages at peak HBV-DNA level and HBeAg seroconversion, y[d]	3.9 ± 3.8
HBV-DNA level at enrollment, $\log_{10}$ copies/mL	8.4 ± 1.0
Peak HBV-DNA level, $\log_{10}$ copies/mL[e]	9.1 ± .9
HBV-DNA level at final follow-up evaluation, % (no.)	
$<10^2$ copies/mL	29.3% (17)
10^2-10^4 copies/mL	36.2% (21)
$>10^4$ copies/mL	34.5% (20)

NOTE. Mean ± SD shown except where indicated.

[a]The ages at HBeAg seroconversion between those with positive and negative maternal HBsAg did not differ (Student *t* test, $P = .22$).

[b]Calculated as the age at HBeAg seroconversion minus the the age at peak ALT level.

[c]Calculated as the age at peak ALT level minus the age at peak HBV-DNA level.

[d]Calculated as the age at HBeAg seroconversion minus the age at peak HBV-DNA level.

[e]The peak HBV-DNA level is higher than the level at enrollment (Student *t* test, $P = .001$).

Table 3. Basic Characteristics, ALT Levels, and Precore Mutants After Spontaneous HBeAg Seroconversion in 58 Seroconverters

	Low viremia	Fluctuating high viremia	*P*
Male:female	23:12	12:11	.30
Mother HBsAg positive (%)	9/35 (25.7)	4/23 (17.4)	.48
Age at enrollment, y	7.0 ± 3.4	7.0 ± 4.1	.99
Final age, y	23.0 ± 3.1	24.9 ± 4.0	.09
HBeAg seroconversion age, y	18.3 ± 4.8	15.7 ± 6.9	.09
Genotype B:C	33:2	20:3	.33
Follow-up duration after seroconversion	4.6 ± 3.8	9.2 ± 5.5	.0005
Median peak ALT level, U/L (range)	24 (14–64)	28 (15–91)	.80
Abnormal ALT level (%)	6/35 (17.1%)	6/23 (26.1%)	.41
Precore 1896 mutant	8/35 (22.9%)	14/23 (60.9%)	.004
Peak HBV-DNA level, $\log_{10}$ copies/mL	3.9 ± 1.6	5.5 ± 1.0	.0001
Lowest HBV-DNA level			.03
$<10^2$, copies/mL, % (no.)	54.3% (19)	21.7% (5)	
10^2-10^4, copies/mL, % (no.)	45.8% (16)	69.6% (16)	
$>10^4$, copies/mL, % (no.)	0	8.7% (2)	

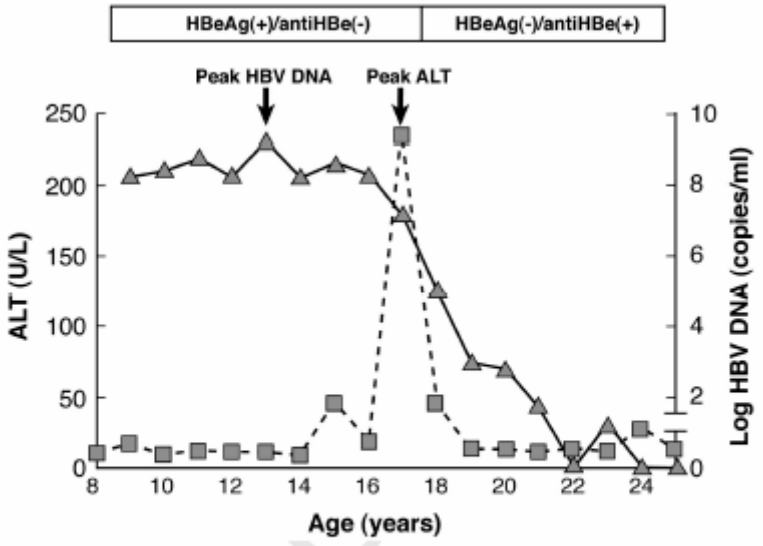

Figure 1. Typical time sequence of the peak HBV-DNA level, peak ALT level, and HBeAg seroconversion in the natural history of chronic HBV infection acquired perinatally. The solid line represents the serum HBV-DNA levels and the broken line represents ALT levels.

PART II . UNIQUE HEPATITIS B GENEONS ISOLATED FROM CHILDREN WITH CHRONIC HEPATITIS B VIRUS INFECTION AND

HEPATOCELLUALR CARCINOMA

Introduction

Chronic HBV infection is a grave issue of public health in hyperendemic regions such as Taiwan and other countries, since it can cause severe complications like liver cirrhosis and hepatocellular carcinoma (HCC) (1). Although human beings have known well the natural course of HBV infection, we still have limited knowledge of the factors contributing to variable clinical course and outcome in different patients with chronic HBV infection. Since most chronic HBV infection begins during infancy or childhood (2) and tends to cause persistent infection (3) and more serious complications (4) than adults, the study in the pediatric patients is thus very important for identifying contributing factors of unfavorable outcome. The HBV genome is susceptible to mutation due to the spontaneous error of its reverse transcriptase (Gunther, 1999) and the lack of proofreading activity of the polymerase. As a consequence of a complex interaction of virus and host immune system, hot spots of mutation may gradually develop during the course of chronic infection. Of our special interest is the correlation between these hot spots of mutation and distinct clinical outcome of chronic HBV infection. In previous studies (5, 6, 7), it has been discovered that some specific HBV genotypes and mutations in the HBV genome have significant impact on the clinical course or outcome of chronic childhood HBV infection. For example, we have also found that genotype B dominates in children with both chronic HBV infection and HCC in Taiwan, while genotype C delays HBeAg seroconversion (6). It has also been found that children with earlier emergence of precore stop codon mutant (G1896A) tend to have more severe hepatocellular damage (5) and this mutant accounts for a half of childhood HBeAg seroconversion (7). In the core promoter region, higher rates of A1775G and G1799C mutations and a lower rate of A1752G mutation have been found in childhood HBeAg seroconverters (7). In the core gene region, children with HCC have more mutations than those with chronic HBV infection; the former group has mutation hotspots at core gene codon 74, 87, and 159, while the later has mutation hotspots at core gene codon 21, 65, and 147 (8). However, previous studies have only focused on limited hot-spot domains, but a systemic analysis of the mutation status of the whole HBV genome and the establishment of its possible clinical relevance have not been achieved. Therefore, the purpose of this study aims at investigating whether a genome-wide comparison and phylogenetic analysis of HBV sequences isolated from children with different courses of chronic HBV infection, including those with HCC, can generate useful information pertaining to region or

sequence features that are specifically related to different consequences of chronic HBV infection in children. The knowledge obtained in this study will greatly enhance our ability of predicting the outcome of chronic HBV infection and promote early therapeutic intervention for certain high-risk patients.

Patients and Methods

Collection and grouping of patients

a) Children with chronic HBV infection

We followed up 415 HBsAg carrier children who were less than 15 years longitudinally for liver function profiles and HBV markers. If their alanine aminotransferase (ALT) levels increased up to more than 2 times of the upper limit of the normal value, monthly determination of HBeAg/anti-HBe and ALT levels were performed until normalization of ALT was reached. We performed full-length HBV sequencing using the blood from 21 age- and sex-matched children who were divided into 3 groups. They were selected according to the following criteria. For group 1, 7 children were randomly selected among HBeAg seropositive patients with acute exacerbation and a peak ALT > 400 IU/L followed by a subsequent HBeAg seroconversion. For group 2, 7 children will be randomly selected from initially HBeAg seropositive patients with a peak ALT level of < 200 IU/L, followed by subsequent HBeAg seroconversion. For group 3, 7 children were randomly selected from patients remaining HBeAg positive without seroconversion during follow-up. For patients in group 1 and group 2, three time points of blood (enrollment, peak ALT levels, and final serum) were analyzed for full-length HBV DNA sequences (totally 21 blood samples for both groups). For group 3, only the initial and final blood samples were subjected to analysis for full-length HBV DNA sequences.

b) Children with HCC

From our HCC blood bank, we randomly selected 7 children with HBV-related HCC whose first diagnoses were made before the age of 15 years. The follow-up period ranged from 1 month to 24.5 years. All except one blood sample, which was obtained 9 years after the diagnosis of HCC, were withdrawn after the diagnostic confirmation of HCC.

Determination of HBV genotypes

The serum samples at enrollment and the latest serum sample available were submitted for HBV genotype analysis. The determination of HBV genotypes has been prescribed previously (6). Briefly, The HBV genotypes were analyzed by using polymerase chain reaction (PCR) with type-specific primers (9). For those samples in which viral loads were so low that HBV genotype could not be determined experimentally, we employed the viral genotyping tool in the National Center for Biotechnology Information (NCBI) web site (http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi) to determine the genotype.

Extraction of DNA from sera

Serum DNA was extracted from 200µl serum by using QIAamp DNA mini kit (Qiagen Inc, Valencia, CA) by following the instructions from the manufacturer. The DNA was dissolved in 100µl buffer TE.

Amplification of full-length HBV genome.

To amplify the full-length HBV genome, we performed the PCR with 3 different primer pairs as shown in Table1 to yield 3 overlapping sub-genomic fragments that covered the full length of HBV genome. The HBV nucleotides were numbered from the *Eco*RI site. The 50µl PCR mixture contained 10X buffer, 5X enhancer, 50mM $MgCl_2$, and 50XdNTP. The polymerase for PCR was the Combizyme DNA polymerase (InViTek GmbH, Berlin, Germany). The PCR condition was performed as following: 96℃ for 2 min; 75℃ for 5 min; 94℃ for 40 sec, 57℃ for 40 sec, 72℃ for 120 sec, for 10 cycles; 94℃ for 40 sec, 55℃ for 40 sec, 72℃ for 120 sec, for 10 cycles; 94℃ for 40 sec, 53℃ for 40 sec, 72℃ for 120 sec, for 10 cycles; 94℃ for 40 sec, 51℃ for 40 sec, 72℃ for 120 sec, for 10 cycles; and finally 72℃ for 10 min. However, the primer pairs described above could not amplify some samples with very low viral loads. Thus, we designed other primers as shown in Table 2 for low viral load samples and the same PCR condition to amplify the full-length HBV sequence. The PCR products were isolated by the electrophoresis and were purified from the 1.5% agarose gel by Gel/PCR DNA Fragments Extraction Kit (Geneaid biotech Ltd. Taiwan).

Cloning of PCR products and isolation of plasmid DNA

The purified PCR products were subjected to cloning using the TOPO XL Cloning Kit (Invitrogen/Life Technologies, Carlsbad, CA) and transformed into *Escbericbia coli* cultured on LB plate containing 50 µl/ml kanamycin. The

recombinant clones were selected and the plasmid DNA was extracted with Mini-M Plasmid DNA Extraction System (Viogene Biotek corp. Taiwan). The DNA samples were dissolved in the 50μl elution buffer. Finally, each plasmid DNA was confirmed by enzyme digestion with EcoR1 (New England Biolabs GmbH, Frankfurt, Germany).

Sequencing and assembling of the full-length of HBV genome, sources of adult HCC full-length sequences, construction of consensus sequences, and phylogenetic analysis

We performed DNA sequencing using M13 forward and reverse primers by ABI DNA sequencer (model3730, version 3.2, Applied Biosystems). We performed the assembling and analysis of HBV genome using the software SeqWeb Version 3.1 (Accelrys Software Inc.). We obtained 48 full-length HBV nucleotide sequences from three groups of sequences submitted to GenBank database. The accession numbers of the first group were AB014360-14390 (10); the numbers of the second group were AB241109, AB241113, AB241114, AB241116, and AB241117 (11); the numbers of the third group were AB113875, AB113876, and AB113877 (published only in GenBank database by Michitaka et al., 2004). The consensus sequences of HBV genomes from patients with either chronic HBV infection or adult HCC were constructed using the software SeqWeb 3.1 supplied by National Health Research Institute (Miaoli County, Taiwan). We carried out phylogenetic analysis by using Phylip version 3.6 provided by National Health Research Institutes (Miaoli County, Taiwan). We used Neighbor-Joining method to do phylogenetic analysis. To evaluate the statistical support, we performed 1,000 bootstrap replications.

Ethical considerations

The blood samples of patients in this study were withdrawn after we obtained the written informed consents from parents of the patients or patients themselves. The Ethical Committee of the National Taiwan University Hospital approved the protocol of study. All sera in this study were processed to protect personal information.

Statistic analysis

The frequency of mutation for each sub-genomic region was calculated by dividing the number of mutation by the number of base pairs of the region. The

frequencies of the highest one and the second highest one were then compared by a chi square test with Yates' correction.

RESULTS

Clinical and laboratory data of patients

The clinical and laboratory profile of three groups (HBeAg seroconverters with high or low peaking ALT levels, and HBeAg non-seroconverters) of patients with chronic HBV infection and 7 children with HCC are listed in Table 3 and Table 4, respectively. Totally 63 HBV full-length sequences were amplified, cloned, and sequenced successfully. Among them, 7 were from children with HCC and 56 were from 21 HBV chronically infected children whose blood samples were withdrawn 2 (enrollment and final follow-up) or 3 (enrollment, peak ALT, and final follow-up) times. In addition, we also retrieved 48 HBV genomes from sera of adult HCC published in GenBank for comparison.

Phylogenetic features of HBV genomes from different clinical settings

In the phylogenetic tree (Fig. 1) based on 111 full-length HBV nucleotide sequences, the HBV genomes from children HCC (H1-H7) were clearly diverged from those from patients with chronic HBV infection and were related to those from adult HCC. Moreover, HBV genomes from patient H1, H2, H3, H6, and H7 were more closely related to each other than to those from H4, and H5. The genomes of H1, H2, H3, and H7 were clustered on a branch that was separated from those of adult HCC patients with a significant bootstrap value (86 %). Although H6 sequence was classified as a neighboring branch to the one consisting of H1, H2, H3, and H7, indeed it was classified in a same cluster as H1, H2, H3, and H7 when only adult HCC isolates were included with 7 childhood HCC isolates to construct the phylogenetic tree (data not shown). To further minimize the region responsible for this classification, we constructed other phylogenetic trees (data not shown or supplementary data) using nucleotide sequences from 7 sub-genomic regions of HBV alone, including those encoding pre-S1 region, pre-S2 region, S region, and the region encoding the large S protein (including the pre-S1, pre-S2, and S), the P gene, the precore/core gene, and the X gene. A similar topology with high relatedness of H1, H2, H3, and H7 was maintained within the regions of pre-S1, pre-S2, and the large S protein, but not in the regions of core and X genes. For the S region and the P gene regions, the topology was loosely maintained, with H1, H2, and H3 still clustered together, but their relationship with H4, H5, H6, and H7 changed. On the other hand, isolates of different groups of children with chronic HBV infection sampled at different time points did not form any cluster with significant bootstrap values (Fig. 1, bootstrap values less than 60% were not shown).

The nucleotide and amino acid characteristics of HBV genomes of H1, H2, H3, H6, and H7

Mutations of the pre-S and the S genes

Since HBV genomes of H1, H2, H3, H6, and H7 share more relatedness, we first focused our analysis on the sequence characteristics of these 5 isolates. By aligning these 5 genomes with the consensus sequence deduced from 56 HBV isolates of chronic HBV infection and with the consensus sequence deduced from 48 adult HCC, we identified some unique nucleotide positions with non-synonymous mutations or deletions (Table 5) in these 5 isolates. These mutations were chosen only when they caused amino acid substitutions in both consensus sequences from chronic HBV infection and adult HCC. In the pre-S1 region, we detected an early stop codon mutation (W4STOP) at nucleotide 2859 position in H1 sequence, which might abolish the synthesis of most of the pre-S1 domain. A similar stop codon mutation located more distally (nucleotide 3055) in the pre-S1 gene has been reported in patients with exacerbation of chronic HBV infection (Minami et al, 1993). The other pre-S1 mutation was found in the amino half of all 5 childhood HCC isolates. In the pre-S2 region, notably, the 4 isolates from childhood HCC (H1, H2, H3, and H7) had a same 12 base-pair deletion (nucleotide 44-55, pre-S2 amino acid, aa 19-22) in the pre-S2 region (Fig. 2). The range of deletion was smaller than those reported in a largest series of study (13). This deletion affected both the epitopes of B cells (aa 1-26) (14) and T cells (aa 21-30) (15) in the pre-S2 region. Other 6 non-synonymous mutations were found in the pre-S2 region. In the S gene, 4 patients (H1, H2, H3, and H7) shared 4 common non-synonymous mutations (G44E, P46Q, and T/V47K, which was generated by mutations of both nucleotide 293 and 294) located within a cytotoxic T lymphocyte (CTL) epitope region (aa 28-51) (16). Another 3 amino acid substitutions (S34L, F41S, T/V47A) also occurred in this region (Table 5), but only in one single patient individually. As for the major hydrophilic region (aa 99-169) (17) of HBsAg, 2 non-synonymous mutations (C121Y and S154P) were found in H7 and H2 sequences, respectively; neither of them was located in the region of immunodominant “a” determinant (18) of HBsAg.

Mutations of Precore/Core Genes

The analysis in the precore region showed that 2 reported mutations (G1862T and G1896A) (19-21) coexisted in 4 childhood HCC isolates (H1, H2, H3, and H7). Both G1862T (19) and G1896A (19, 21) mutations are associated with reduced capacity of HBV to produce hepatitis B e antigen (HBeAg) from its precore protein

precursor. The G1896A mutation is also associated with severe liver damage in patients with chronic hepatitis B (22). In the core gene, it is remarkable that all 5 childhood HCC patients (H1-3, H6, and H7) shared 2 mutations at nucleotide 2048 (P50A) and 2073 (A58E) in a region of CD4+T cell epitope (aa 48-69) (23). The mutation at nucleotide 2048 (core gene codon 147) is one of the 3 most frequently occurred mutation in chronic HBV infected children (8). For other known epitopes, there was 1 non-synonymous mutation (V13A) in the HLA-A2-restricted CD8+ CTL epitope (aa 18-27) (24-5) in isolates from patient H1, H2, H3, and H7, while there were 2 non-synonymous mutations (T147A and R151Q) in HLA-Aw68-restricted CTL epitope (aa 141-151) (25) in patient H1, H3, H6, and H7. Within the hot-spot mutational domain (aa 80-120) reported to be related to severe liver diseases (26-28), we found 3 non-synonymous mutations (A80T, L84A, and Y118F) in patient H1, H3, H6, and H7.

Mutations of polymerase and X genes

The analysis of the polymerase (pol) gene of 5 isolates showed that the majority of non-synonymous mutations (Table 5) were located in the reverse transcriptase (RT) (nucleotide 130-1161) and spacer domains (nucleotide 2838-129) (29). As in the pre-S2 region, there was a 4-amino acid deletion in the spacer domain of isolated from patient H1, H2, H3, and H7. In the RT domain of isolates from patient H1 and H2, there was a mutation at nucleotide position 880 (aa 251 of the RT gene) that changed glycine to a stop codon (G251STOP) and generated a truncated RT lacking both the c-terminal region of the RT domain and the RNase-H domain (nucleotide 1152-1623) (29). In the RNase-H domain, the isolate from patient H6 carried a non-synonymous mutation at nucleotide 1613 (rhR240K or R841K), which also cause a synonymous mutation in X gene open reading frame known to have a higher relative risk of HCC development (30). In addition, there were 2 mutations located in 2 HLA class I restricted T-cell epitopes of the pol gene (31): nucleotide 760 (A557S, patient H6 and H7) of YMDDVVLGA (aa 549-557), and nucleotide 828 (I579M, patient H1) of FLLSLGIHL (aa 573-581). Our 5 isolated did not carry the well-known rtM204I/V mutation in the YMDD motif (32) or other mutations in the pol gene associated with antiviral therapy (33-34). The analysis of X gene region revealed only 4 non-synonymous mutations in isolated from patient H1, H2, H3, and H7 (Table 5). We did not find other mutations or deletions in the X gene reported previously in patients with severe liver diseases (35) or with HCC (30, 36). Since parts of sequences in this region also function as core promoter and other regulatory elements, the mutations within them will be discussed in the following paragraphs.

Mutational frequencies of different regions of the HBV genome

We compared the frequencies of non-synonymous mutations in different regions of HBV (pre-S1, pre-S2, S, precore, core, pol, and X genes) (Table 6) and found that the pre-S2 region had the highest mutation frequency (compared with that of the second highest one, the core gene, $X^2 = 16.677$, $P < 0.0001$).

Mutations of the regulatory regions of HBV

In addition to the non-synonymous mutations, we also found 14 synonymous mutations in HBV genomes of the isolates from 5 children with HCC (Table 7). The analysis of both groups of mutations in the regulatory sequences of HBV showed that some of them might change the important regulatory sites. In the core promoter/enhancer II region, the clustered mutations at nucleotide 1632, 1633, and 1634 (Table 8) of isolates from patient H1, H2, H3, and H7 were immediately next to a Sp1 site (nucleotide 1622-1631) (37). In addition, the HBV genomes of these 4 patients also had 2 mutations (nucleotide 1482 and 1566, Table 8) located in the negative regulatory element gamma and alpha (38), respectively. In the basal core promoter (BCP), the well-known BCP (A1762T, G1764A) mutation that increases that risk of HCC in HBV carries (36) existed in isolates from patient H4, H5, H6, and the majority of HCC adults, but not in those from patient H1, H2, H3, and H7. In the region of S1 and S2 promoters, 4 mutations (Table 8) were noted but were not close to any known transcription binding sites. In the enhancer/X promoter regions, we found 7 mutations (Table 8). Notably, 3 of them (nucleotide 1120, 1123, and 1128, all from patient H7) were located in the hepatocyte nuclear factor 3 (HNF3) binding site (nucleotide 1120-1129) (39). In addition, the retinoic acid responsive element (RARE, nucleotide 1136-1148) (40) in enhancer I of the isolate from patient H7 harbored one mutation (nucleotide 1138).

Discussion

Our phylogenetic analysis of the HBV genomes from children either with HCC or chronic HBV infection at different clinical settings, and from adult HCC available in GenBank indicated the emergence of a unique and childhood HCC-related cluster of HBV genomes. It suggested that selection pressure might be great enough in these patients to cause the genetic differentiation of HBV virus. In addition, the direct sequence comparison among these 3 groups of HBV genomes revealed several characteristic mutations/deletions specific to childhood HCC group. The most striking one may be the consensus 12-bp in-frame pre-S2 deletion in 4 out of 7 cases. Recently several studies have shown significant association of pre-S deletions of HBV genomes with severe liver diseases, including HCC (13, 41-45). However, the reported pre-S deletions involve both the pre-S1 and pre-S2 regions but only the pre-S2 deletion was found in our cases. In HBV genomes from adult HCC, Chen et

al. (13) have identified multiple patterns of pre-S deletion. Our deletion, which was located in the N-half of the pre-S2 gene, can be classified as type V deletion according to their system. Type V deletion comprises 29.3% of pre-S deletions found in adult HCC. Interestingly, 75% of their type V deletions are larger in length than our deletion and 58.3% of type V deletions contain our 12-bp deletion region. Therefore, it is probable that the deletion we found defines a smaller critical region, which when deleted may be linked to HCC pathogenesis. How does the internal pre-S2 deletion in HBV contribute to the development of HCC? One possibility is that it may compromise both B- and T-cell epitopes of the pre-S2 region (14, 15) and escape the immune-mediated viral clearance. Other plausible mechanisms include that the large surface protein containing the pre-S2 deletion (so called Delta S2-LHBs) may induce endoplasmic reticulum stress (46) that exerts genotoxic effects leading to cancer formation and that the Delta S2-LHBs can upregulate cyclinA expression and hepatocyte proliferation (47). It remains to be determined whether pre-S2 deletion is sufficient to generate real liver cancers in vivo. For confirming the role of pre-S2 deletion in HCC oncogenesis, it may be also necessary to screen the pre-S2 deletion in a larger cohort with chronic HBV infection and determine the relative risk of HCC development prospectively in those whom harbor these deletions.

The coexistence of both G1862T and G1896A precore (PC) mutations found in HBV genomes from 4 of 7 childhood HCC is consistent with previous reports showing that both mutations were associated with severe liver diseases and HCC (19-21). Interestingly, HBV genomes of these 4 patients also harbored the consensus 12-bp pre-S2 internal deletion. In contrast, we did not detect the well-known BCP (A1762T, G1764A) mutations (36) in these 4 cases, although BCP mutations were present in the majority of HBV genomes from adult HCC we obtained from GenBank and in one out of 7 isolates from group 1 chronic HBV-infected patients who had higher peak ALT. Recently it has been reported (13) that in HBV genomes of adult HCC, pre-S2 deletions rarely exist alone and coexist frequently with PC rather than with BCP mutations. Our results are in agreement with it and we did not find co-existence of BCP mutations and pre-S2 deletions in any case.

In other regions, we also found unique mutations of HBV genomes from childhood HCC that are rarely reported. Some of them may alter the immune response of the host against the virus, since they were located in the epitopes of B or T cells. For example, in the of the majority of childhood HCC-related isolates, we found G44E, P46Q, and T/V47K in a CTL region (aa 28-51) (16) of the small surface protein, P50A and A58E in a CD4+ T cell epitope (aa 48-69) (23) of the core protein, and T147A and R151Q in a HLA-Aw68-restricted CTL epitope (aa 141-151)

(25) in the core protein. Other less frequently occurred mutations include A557S and I579M (Table 5) in class I restricted T-cell epitopes of polymerase (31). In addition, the childhood HCC-related HBV genomes also contained mutations in previously reported regions where mutations are associated with severe liver diseases and HCC (26-28, 30), such as aa 88 (R841K) (30) in the polymerase and the hot-spot mutational domain (aa 80-120) (26-28) of the core protein (Table 5). Finally, in the P gene, we found several mutations in the domains of polymerase that interacts heat shock protein 90 (Hsp90) (48), including one common mutation (A137T, all 5 patients) in the C terminal domain (after aa 97) of the terminal protein (TP), and a premature stop codon mutation (G597STOP, patient H1 and H2) that abolishes the expression of the thumb region of RT domain (aa 616-680). By interacting with these 2 domains of polymerase, Hsp90 can associate with RT and facilitate the folding of RT into an active form. However, it remains to be determined to what extent the mutations change the interaction and what the biological consequences of the change are.

HBV mutations accumulate gradually during long-term chronic infection. What cause the early emergence of HBV mutants that possibly linked to HCC development in these children? So far the answer is still vague, although the interplay of genetic, immune, and environmental factors may be responsible for the pathogenesis. Most of these patients came to our clinics with initial manifestation of HCC without regular follow up in hospitals. However, we clearly know that they were likely infected vertically since all the mothers were HBV carriers. All patients were HBeAg seroconverters before the detection of HCC. No significant difference was seen in their HBV genotypes (B: C = 3: 4) although the cohort is small. Interestingly, among these 7 patients, we found one whose blood analyzed was withdrawn before he developed detectable HCC, suggesting that these unique genomes may exist in the liver previously and may possibly participate in the process of carcinogenesis instead of merely being a biomarker of HCC. To confirm this hypothesis may require incorporation of more HBV genomes from both children and adults into our databank for phylogenetic analysis and a well-designed prospective study that compares the clinical outcome between patients with distinct HBV genomes.

In conclusion, we identified unique HBV genomes in children with HCC. These HBV genomes share a 12-bp pre-S2 deletion and several characteristic mutations that when in combination may be possibly related to early development of HCC. Moreover, our results indicated that analysis of the whole genome of HBV in more patients with chronic HBV infection may be helpful, especially when phylogenetic analysis can be performed together with known HBV genomes of both

mild and advanced liver diseases, since this comprehensive approach may identify unique HCC-related HBV genomes and those who harbor them may benefit greatly from early detection and therapeutic intervention of advanced diseases.

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Table 1 The primers used for PCR

Fragment 1	(HBV nt 1821-2516)	
P1	(forward, nt 1821-1844)	5' –TTTTTCACCTCTGCCTAATCATCT– 3'
NP111038A	(reverse, nt 2516-2497)	5' –AAAGACAGGTACAGTAGAAG– 3'
Fragment 2	(HBV nt 427-1825)	
NP210101A	(forward, nt 427-448)	5' –CATCTTCTTGTTGGTTCTTCTG– 3'
P2	(reverse, nt 1825-1804)	5' –AAAAAGTTGCATGGTGCTGGTG– 3'
Fragment 3	(HBV nt 2268-479)	
NP3	(forward, nt 2268-2291)	5' –GAGTGTGGATTCGCACTCCTCCAG– 3'
NP4	(reverse, nt 479-456)	5' –GAGGACAAACGGGCAACATACCTT– 3'

Table 2 The primers used for PCR with low viral-load samples

Fragment 1	(HBV nt 1573-2516)	
NP1SF	(forward, nt1573-1594)	5' – GACCGTGTGCACTTCGCTTCAC – 3'
NP111038A	(reverse, nt 2516-2497)	5' – AAAGACAGGTACAGTAGAAG – 3'
Fragment 2	(HBV nt 542-1741)	
NP2SF	(forward, nt 542-564)	5' –GGAAACTCTATGTTTCCCTCATG– 3'
NP2SR	(reverse, nt1741-1719)	5' –AACTCCTCCCACTCAKTAAACAC– 3'
Fragment 2-1	(HBV nt 488-1188)	
HCC 2-1F	(forward, nt 488 - 507)	5' – GAAACATCAACTACCAGCAC– 3'
HCC 2-1R	(reverse, nt 1188-1169)	5' – AGCAAACACTTGACAGAGAC– 3'
Fragment 2-2	(HBV nt 1136-2098)	
HCC 2-2F	(forward, nt 1136-1155)	5' – TGAACCTTTACCCCGTTGCC– 3'
HCC 2-2R	(reverse, nt 2098-2078)	5' –CATCAACTCACCCCAACACAC– 3'
Fragment 3	(HBV nt 2528-704)	
HCC 3F	(forward, nt 2528-2549)	5' – GCAAACTCCCTCCTTTCCTCAC– 3'
HCC 3R	(reverse, nt 704-683)	5' – CGAACCACTGAACAAATGGCAC–3'

Fragment 3	(HBV nt 2268-690)	
NP3	(forward, nt 2268-2291)	5’ –GAGTGTGGATTCGCACTCCTCCAG –3’
NP410004C	(reverse, nt 690-671)	5’ –AATGGCACTAGTAAACTGAG– 3’

Table 3. Basic data of three groups of patients with chronic HBV infection

	Group 1	Group 2	Group 3
Gender (M:F)	4:3	4:3	4:3
Age at enrollment (yr)	8.0 ± 2.8	7.5 ± 2.4	8.1 ± 1.7
Follow-up duration (yr)	17.2 ± 1.9	16.7 ± 1.3	16.0 ± 1.0
Initial ALT level (*U/L*), mean ± SD	11 ± 2	12 ± 5	12 ± 6
Peak ALT level (*U/L*), mean ± SD	614[a, b] ± 297	91[a] ± 50	55[b] ± 42
Final ALT level (*U/L*), mean ± SD	18 ± 7	21 ± 21	29 ± 9
Virus genotype (B:C)	6:1	6:1	6:1
HBeAg seroconversion (%)	100	100	0
Mother as HBsAg carrier (%)	100	85.7	85.7

[a]: The peak ALT levels between group 1 and 2 showed significant difference ($P < 0.001$), Mann-Whitney test; [b]: The peak ALT levels between group 1 and 3 showed significant difference ($P < 0.001$), Mann-Whitney test.

Table 4. Clinical profiles of 7 children with HCC

	H1	H2	H3	H4	H5	H6	H7
Gender	F	M	M	M	M	M	M
Age with initial tumor	6.4	7.6	8	10	10.4	14.2	11.3
Age at blood sampling	6.9	7.6	9	22.4	10.5	5.3	34.6
Age at final F/U	9.1	14.3	9.4	24.8	11	14.2	35.8
Live/Dead	L	L	D	L	D	D	L
AST/ALT	31/32	377/522	62/33	38/46	166/86	29/15	18/27
HBsAg/Anti-HBsAb	(+/-)	(+/-)	(+/-)	(+/-)	(+/-)	(+/-)	(+/-)
HBeAg seroconversion	(+)	(+)	(+)	(+)	(+)	(+)	(+)
Maternal HBsAg	(+)	(+)	(+)	(+)	(+)	(+)	(+)
HBV genotype	C*	C*	C	B	C*	B	B
AFP level (ng/ml)	41811	10621	-	>70,000	>87,500	108.4	>1600

H1-7: HCC patients No.1-No.7; F/U: follow-up; *: cases whose viral genotypes determined by sequence analysis only due to insufficient blood amount; AFP: alpha-fetopprotein

Table 5: Unique non-synomonous mutations found in 5 childhood HCC full length HBV sequences

Nucelotide level					Protein level	
location[a]	origin[b]	changed	percentage	patient No.	P gene	Pre-S2 gene
8	A	G	20%	H2	N306S	T7A
30	A	G	20%	H7	syn	D14G
40	A	G	100%	H1,2,3,6,7	K317E	syn
44	G	DEL	80%	H1,2,3,7	G318D	G19DEL
45	G	DEL	80%	H1,2,3,7	G318D	G19DEL
46	C	DEL	80%	H1,2,3,7	P319DEL	G19DEL
47	C	DEL	80%	H1,2,3,7	P319DEL	L20DEL
48	T	DEL	80%	H1,2,3,7	P319DEL	L20DEL
49	A	DEL	80%	H1,2,3,7	I320DEL	L20DEL
50	T	DEL	80%	H1,2,3,7	I320DEL	Y21DEL
51	A	DEL	80%	H1,2,3,7	I320DEL	Y21DEL
52	C	DEL	80%	H1,2,3,7	L321DEL	Y21DEL
53	T	DEL	80%	H1,2,3,7	L321DEL	P22DEL
54	T	DEL	80%	H1,2,3,7	L321DEL	P22DEL
55	T	DEL	80%	H1,2,3,7	S322DEL	P22DEL
56	C	A	80%	H1,2,3,7	S322DEL	P23T
60	C	T	80%	H1,2,3,7	syn	A24V
122	A	G	80%	H1,2,3,7	N344S	I45V
						S gene
162	A	G	20%	H6	syn	N3S
255	C	T	20%	H2	syn	S34L
276	T	C	20%	H1	syn	F41S
285	G	A	80%	H1,2,3,7	syn	G44E
291	C	A	80%	H1,2,3,7	syn	P46Q
292	C	A	80%	H1,2,3,7	H/R401K(H1,2,3,7)	syn
293	A/G	A	80%	H1,2,3,7	H/R401K(H1,2,3,7)	T/V47K(H1,2,3,7)
	A/G	G	20%	H6	H/R401R(H6)	T/V47A(H6)
294	C/T	A	80%	H1,2,3,7	H/R401K(H1,2,3,7)	T/V47K(H1,2,3,7)
	C/T	C	20%	H6	H/R401R(H6)	T/V47A(H6)
357	T	C	20%	H6	syn	I68T
369	A	G	20%	H7	syn	Y72C
381	G	A	20%	H6	syn	C76Y

429	T	C	80%	H1,2,3,7	syn	I92T
445	T	C	20%	H1	S452P	syn
499	T	C	60%	H1,2,7	Y470H	syn
511	A	G	80%	H1,2,3,7	T474A	syn
516	G	A	20%	H7	M475I	C121Y
517	C	T	20%	H3	Q476T	syn
532	T	C	20%	H1	S481P	syn
617	T	C	20%	H2	I509T	S154P
709	G	A	20%	H3	A540T	syn
760	G	T	40%	H6,7	A557S	syn
828	A	G	20%	H1	I579M	Y225C
880	G	T	40%	H1,2	G597STOP	
898	T	G	20%	H3	W603F	
934	A	C	60%	H1,2,3	I615L	
936	C	T	20%	H3	I615L	
1083	G	C	20%	H6	K664N	
1123	T/A	C	20%	H7	C/S678R	
1128	A	C	20%	H7	K679N	
1138	A	C	20%	H7	N683H	
						X gene
1482	C	G	80%	H1, 2, 3, 7	L797	L37V
1566	T	C	80%	H1,2,3,7	H825R	S65P
1613	G	A	20%	H6	R841K	syn
1632	C	G	80%	H1, 2, 3, 7		Q/A87G
1633	A/G	G	80%	H1, 2, 3, 7		Q/A87G
						Precore gene
1862	G	T	80%	H1, 2, 3, 7		V17F
1896	G	A	80%	H1, 2, 3, 7		W28Stop
						Core gene
1938	T	C	80%	H1, 2, 3, 7		V13A
2001	C	T	20%	H6		A34V
2003	T	A	80%	H1, 2, 3, 7		S35T
	T	C	20%	H6		S35L
2004	C	T	20%	H6		S35L
2007	C	A	20%	H6		A36D
2010	T	G	20%	H6		L37R
2048	C	G	100%	H1, 2, 3, 6, 7		P50A
2073	C	A	100%	H1, 2, 3, 6, 7		A58E

2138	G	A	80%	H1, 3, 6, 7		A80T
2150	T	G	80%	H1, 3, 6, 7		L84A
2253	A	T	80%	H1, 3, 6, 7		Y118F
					P gene	
2339	A	G	80%	H1, 3, 6, 7	syn	T147A
2352	G	A	80%	H1, 3, 6, 7	D16K	R151Q
2366	C	G	80%	H1, 3, 6, 7	syn	P156A
2423	A	C	80%	H1, 3, 6, 7	E39D	syn
2525	G/A	T	20%	H2	E73D	
2547	C/G	A	40%	H6,7	H81N	
2563	A	G	100%	H1,2,3,6,7	E86G	
2715	G	A	100%	H1,2,3,6,7	A137T	
						Pre-S1 gene
2859	G	A	20%	H1	V185I	W4STOP
2991	C	A	100%	H1,2,3,6,7	Q229K	N48K
3063	C/G	A	100%	H1,2,3,6,7	R/G253R	syn
3165	T	C	20%	H2	S287P	syn

[a]: nucleotide position counted from the EcoRI site; [b]: when two characters shown and separated by a slash, meaning that the former was from adult HCC group and the later was from children with chronic HBV infection; syn: synonymous mutations; DEL: deletion; underlined characters: meaning that the amino acid mutation was generated together by the one underlined and previous 1 or 2 nucleotide positions.

Table 6: Frequencies of non-synonymous mutations of different HBV regions

Region	Frequency (mut No./bp)
Pre-S1	0.0056
Pre-S2	0.1030*
S	0.0235
Precore	0.0230
Core	0.0272*
Polymerase	0.0221
X	0.0086

mut. No.: number of mutation; bp: number of base pair; * $P < 0.0001$ (chi square test with Yates' correction

Table 7 Synonymous mutations in HBV genomes of 5 children with HCC

location[a]	origin	changed	patient No.	genes/regulatory regions
927	A	C	H3	pol
969	A	G	H6, 7	pol/enhancer I
1120	C	T	H7	pol/enhancer I
1167	A/T	C	H1, 2, 7	pol/enhancer I
1634	G	A	H1, 2, 3, 7	pol/enhancer II
1933	T	C	H1, 2, 3, 7	core
2005	T	C	H6	core
2035	G	T	H1, 2, 3, 6, 7	core
2074	T	G	H1, 2, 3, 6, 7	core
2080	G	A	H1, 6, 7	core
2095	G	A	H1, 3, 6, 7	core
2128	G	A	H1, 3, 6, 7	core
2151	T	C	H1, 3, 6, 7	core
2561	G	A	H1, 2, 3, 6, 7	pol

[a]: nucleotide position counted from the EcoRI site; b: when two characters shown and separated by a slash, meaning that the former was from adult HCC group and the later was from children with chronic HBV infection; also noting that for the regions with two open reading frames (ORF), only the changes caused synonymous mutation in both ORFs were included in this table; otherwise listed in Table 5.

Table 8 Mutations within or near the known regulatory elements

location[a]	regulatory subregions	specific elements
969	enhancer I	nil
1083	enhancer I	nil
1120	enhancer I	HNF (1120-29)
1123	enhancer I	HNF (1120-29)
1128	enhancer I	HNF (1120-29)
1138	enhancer I	RARE (1136-48)
1167	enhancer I	nil
1482	NRE-gamma	nil
1566	NRE-alpha	nil
1632	core promoter	near Sp1 (1622-31)
1633	core promoter	near Sp1 (1622-31)
1634	core promoter	near Sp1 (1622-31)
2715	S1 promoter	nil
2991	S2 promoter	nil
3063	S2 promoter	nil
3165	S2 promoter	nil

[a]: nucleotide position counted from the EcoRI site; HNF: hepatocyte nuclear factors; RARE: retinoic acid responsive element; both synonymous and non-synonymnous mutations included

Figure1.

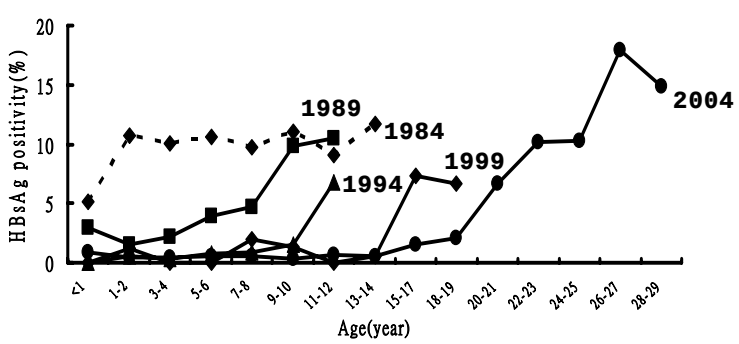

Figure 2.

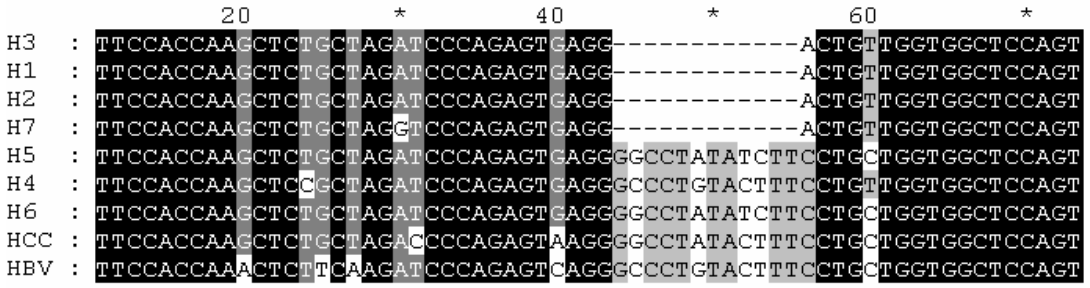

Figure legends:

Figure 1. Phylogenetic classification of 111 HBV genomes from 3 sources: 1) 7 children with HCC, 2) 56 samples of 21 children with chronic HBV infection at different time points, and 3) 48 adult HCC patients (from GenBank). The phylogenetic tree was constructed using Phylip Version 3.6 software. Bootstrap numbers more than 60% are shown. Two large brackets indicate the clusters of isolates of chronic HBV infection of children and adult HCC as labeled. A small brace symbol indicates the position of a special cluster of genomes consisting of isolates from childhood HCC patient H1, H2, H3, and H7. An arrow head shows the location of isolates from childhood HCC patient H4 and H5. "*": the only isolate of adult HCC that was classified close to those of chronic HBV infection. Names of isolates from children with chronic HBV infection (the upper left group) were initiated with "BH" (higher ALT levels), "BL" (lower ALT levels), or "BN" (HBeAg non-seroconverters). Names of isolates from adult HCC (the lower right group) were given as their accession numbers in GenBank initiated with "ab".

Figure 2. The consensus sequence of pre-S2 deletion in isolates from 4 children with HCC The dashed lines mark the location of the 12-bp deletion, HCC: the consensus sequence deduced from isolates of adult HCC by SeqWeb3.1; HBV: the consensus sequence deduced from isolates of children with chronic HBV infection; black-shadowed regions indicate the perfectly conserved regions, while the gray-shadowed regions represent the partially conserved regions.

行政院國家科學委員會補助國內專家學者出席國際學術會議報告

95 年 11 月 3 日

附件三

報告人姓名	張美惠	服務機構及職稱	台大醫學院小兒科
時間 會議 地點	95 年 10 月 25 日至 11 月 1 日 美國波士頓	本會核定補助文號	NSC95-2314-B-002-019
會議名稱	(中文)美國肝臟醫學會 (英文)American Association for the Study of Liver Diseases(AASLD)		
發表論文題目	(中文)以大便顏色卡作全國性膽道閉鎖篩檢 (英文) UNIVERSAL SCREENING FOR BILIARY ATRESIA IN TAIWAN BY STOOL COLOR CARDS		

報告內容應包括下列各項：

一、 參加會議經過

10 月 25 日夜晚出發，10 月 26 日抵達波士頓(Boston),10 月 27 日開始開會。會議自 10 月 27 日至 10 月 31 日。共有各國六千多人參加此國際上肝臟學最重要的學術會議。本人參加之會議行程及內容如下：

I.本人報告的論文"Universal Screening for Biliary atresia in Taiwan by stool color cards"排在 10 月 29 日。

II.北美兒科消化醫學會與美國肝臟學會合辦之兒科肝臟學專題研討會(10 月 27 日)主題為"遺傳代謝性肝疾病之新進展"—由實驗室至臨床"。內容涵括：

1)膽酸代謝異常及肝病

2)粒線體疾病 – 能量與疾病

3)脂肪酸氧化異常與肝病

4)醣化異常 – 醣缺乏醣蛋白徵候群與肝病

5)代謝疾病的肝細胞移植

III.再教育課程：(Postgraduate Course)

1)急性肝傷害及衰竭：(10 月 27 日)

a.肝細胞如何死亡

b.肝傷害的免疫角色

c.急性肝衰竭的原因及預後

d.急性肝衰竭的治療

e.肝傷害的標靶治療

2)病毒性肝炎(10 月 27 日)

a.C 肝病毒如何傷害肝臟
b.目前及未來 C 肝之治療
c.肝移植後 C 肝再發的特徵
d.B 肝病毒如何傷害肝臟
e.目前及未來 B 肝之治療

3)脂肪肝炎：非酒精性及酒精性肝炎(10 月 28 日)
a.脂肪肝是代謝徵候群的一部份
b.脂肪造成肝傷害的機轉
c.非酒精性脂肪肝的未來新療法
d.兒童肥胖之流行病學與脂肪肝病變
e.以類固醇及抗 TNF-α 治療酒精性肝炎的爭議

4)肝再生及移植(10 月 28 日)
a.肝如何再生
b.肝幹細胞的臨床意義
c.部份肝移植:增進臨床肝再生
d.現今及未來的免疫抑制療法
e 活體肝移植之爭議

5)基因學在肝病的角色(10 月 28 日)
a.基因與疾病
b.為何有些人會肝纖維化，有些人不會
c.兒童胆汁滯留症之新觀念
d.孕期膽汁滯留症之基因學及治療
e.改變基因表現之新藥治療

6)肝膽腫瘤(10 月 28 日)
a.肝胆腫瘤之機轉
b.肝胆腫瘤之偵測新境界
c.肝細胞癌之新治療
d.胆管細胞癌之新治療

IV.美國肝臟學會 2006 年年會(10 月 29 日至 31 日)

1)清晨研討會(6:45AM-8:00AM)有許多題目，每日分許多小討論室進行。本人參加下列主題。
a.肝幹細胞生物學(10 月 29 日)由 Gupta 教授等主持，與參與者(大約二十人左右，面對面討論肝幹細胞的定義，分類，標記，臨床意義及用途。
b.蛋白質體學(10 月 30 日)由實驗原理,方法,以討論肝細胞癌新標記的開發為例子，說明標本收集的重要性，及再次驗證其結果的重要性。

2)基礎研究研討會(10 月 29 日上午)主題為"病原體認知之受體"
a.Toll 樣受體(TLR)與肝臟

i)病原體認知受體之概論

ii)Toll 樣受體的敏感化與肝傷害

b.病毒性肝炎類型認知之受體：

i)C 型肝炎的 TLR 及 RIG-1 訊息傳遞

ii)C 型肝炎的 SNP 及本能免疫反應

c.TLR 在肝傷害與肝再生：

i)TLR：聯結本能免疫力及適應性免疫力。

ii)MyD88 及肝再生

iii)TLR 及肝缺血/再灌流傷害。

3)特別演講：本人參加下列演講

a.Hans Popper 基礎科學演講

Crispe IN 教授主講"肝免疫反應：病理學及耐受性"，由肝一般免疫反應談到對內毒素之反應可能是主因，以至耐受性之產生，及為何會發生肝疾病。

b.Hyman Zimmerman 肝毒性演講

Maddrey WC 教授討論 statin 的使用與肝酵素的上升，比較 statin 與其他肝毒性藥物的肝變化，評論 statin 在已有肝疾病患者使用的安全性。

c.Leon Schiff 特別演講

討論止血藥物對於預防及控制消化道出血的輔助效果，探討近年來用以評估血液凝固功能的檢測方法較過去的老方法更能反應人體的血液凝固功能，並評論以止血藥物來輔助門脈高壓及其出血合併症之治療的功效。

4)集合演講(Plenary Session)

5)分堂演講(Parallel Session)：本人參與下列主題

a.幹細胞與細胞生長與死亡之調控

b.兒科肝臟學之新新進展

c.免疫學及細胞移植

6)壁報展示(Poster Session)：本人及同仁均有壁報參展；本人並參與幹細胞及兒科肝臟學之壁報評估工作。

7)其他:尚有下列會議在大會中舉行

a.國際肝移植/美國肝臟學會聯合研討會

b.美國消化道內視鏡學會/美國肝臟學會聯合研討會

c.美國國家衛生院肝臟學會議

d.肝臟學焦點研究群討論會

V.參加 SCI 雜誌"Liver International"編委會:10 月 28 日晚上 6 至 9 時(本人是五位主編之一)。

VI.參加 AASLD 節目評估委員會會議:10 月 30 日晚上 6 至 8 時(本人是 AASLD 節目評估委員會委員)

二.參加會議心得(與會心得)：

I.本人報告論文”Universal Screening for Biliary Atresia in Taiwan by Stool Color Cards.”膽道閉鎖是兒童因肝臟病死亡以及換肝最常見的原因。膽道閉鎖兒若不能在60天大以前接受葛西手術，則其手術後的成功機率會降低，十年存活率也會降低。但過去台灣的患者診斷常常較晚，只有大約36%左右在60天以前完成葛西手術。我們自2002年自局部地區開始推展大便顏色卡篩檢膽道閉鎖。在2004年推展至全台灣。我們發現在逐步推展下，60天大以前接受葛西手術患者由58%逐漸上昇至78%。台灣是世界上第一個施行全國性篩檢膽道閉鎖的國家,我們的報告受到其他國家的注意，美國，英國，馬來西亞等國均考慮參考我們開發的全國篩檢系統以期早期診治他們的膽道閉鎖兒童。

II.學習新知:此次會議中學習許多新的重要的知識與訊息,尤其在肝幹細胞,細胞再生，免疫反應，基因調控，蛋白質體學，新的肝炎治療，脂肪肝的成因及新治療，肝腫瘤之形成機轉等均有很好的收獲。在兒科肝臟學方面，膽道閉鎖的機轉之研究，也有最新的報告。以上的新知，對於我們目前研究的設計及方向的調整均很有助益。

1.肝細胞的死亡調控：

細胞之死亡可能是壞死或凋亡，死亡受體CD95(Fas)啟動訊息傳遞TNF→TNFR至細胞核NFKB→JNK→caspase等造成細胞死亡。肝細胞死亡有種型態，若進行無法控制的大量凋亡及壞死，會走上急性肝衰竭的狀況，若這些細胞存活，可能走上肝細胞癌之路。 Acetaminophen過量，缺血/再灌流B肝病毒句感染等均可導致肝細胞凋亡，而發生急性肝衰竭。肝癌細胞失去了CD95的表現，易生腫瘤Huh7即是一個例子。轉移CD95會回復其細胞凋亡是敏感性。

另一個例子是銅代謝疾病-Wilson病，一個肝細胞殺死別的肝細胞。脂肪性肝炎的動物模式ob/ob鼠之肝細胞對JO2很敏感，很容易進行凋亡，Fas受體有過度表現的現象 。

2.急性肝衰竭機轉及治療

急性肝衰竭(ALF)的原因很多，以動物模式造成急性肝衰竭，可有下列主要的方式：

1) LPS/galactosamine
2) Agonist Fas monoclonal anitibody (FasL)
3) Con A (經T 細胞之作用)
4) 缺血/再灌流
5) 毒物，例如Acetaminophen,ccl4等，這些因子給予(1)肝細胞壓力(stress),釋放ROS,JNK訊息傳遞(stress kinase)，(2)加上TNFα 訊息傳遞增加；(3)本能的免疫反應提升，促使細胞素,chemo kine等釋放,使caspase增加,粒線體膜透性增高等,這是一種惡性循環,造成細胞大量死亡，而使肝衰竭。

*治療可針對這些機轉，使用

1)Pancaspase 抑制劑：

2)將標靶對準粒腺體外膜,使用 MOMP 抑制劑,可對抗細胞凋亡,及降低 proapoptotic 之物質。

3)使用 JNKZ (但非 JNK1)之抑制劑可減少肝傷害及肝衰竭。

4)TL-6 或 HGI 之 agonist 的使用。

5)抑制 TNF-α 。

這些訊息傳遞路徑，可受 ATP 之存在與否而影響。若 ATP 存在，往往走依賴能量的路，亦即 apoptosisi；若 ATP 不存在，往往走壞死的路徑。

在抑制 caspase 時，要注意，可能可以緩解急性肝衰竭，但部份肝細胞可能由凋亡變成壞死。使用 TNF-α 之抑制劑之好壞處也要衡量。

3.肝炎病毒如何傷害肝臟，及其治療：

(1)B 型肝炎：

B 肝病毒傷害肝細胞途徑可分為細胞破壞性及非細胞破壞性兩類。若將 CD8+細胞去除，會延長肝細胞表現 HBV 之核心抗原。在急性 B 型肝炎時細胞毒性 CD8 細胞扮演重要角色造成肝傷害。慢性 B 肝患者則只有微弱而功能不良的 CD8T 細胞，其肝細胞表現 B 肝抗原，有些細胞表現 HBsAg，有些則表現 HBcAg 有相當變化性。部分 CD4 陽性 B 肝病毒特異性細胞，及 NK 細胞的抗病毒作用，會引發漸進性的肝傷害。至於肝移植後的肝炎患者，由於免疫抑制劑之使用，使 CD8 無效能，CD4 及非特異性免疫細胞造成肝傷害，病毒呈現繼續複製的狀況，總之，無病毒，無肝傷害。

B 肝之治療藥物除了 Lamivudine, adefovir,entecavir 21%,placebo~5%,Pegylated interferon 32%, PegIFN 加 lamivudine 為 27% 。至於用藥性產生，lamivudine 一年為 15-30%,5 年為 70%,Aelefovir 一年 0%,五年為 30-40%,enecavir 一年為 0%,二至三年為<1%,Telbivudine 一年為 2-3%。二年為 18%。新藥方面：FTC(emtricitabine)是一種 L-nucleoside, Tenofovir, pradefovir 則為 L-nucleotide,均在人體試驗中。至於 Clevudine(L-FMAu)效果與 aplacebo 很相似。

(2)C 型肝炎：

其細胞凋亡是病毒直接及非直接的作用。HCV 蛋白會活化 caspase 促進細胞凋亡。C 肝病毒核心蛋白(core 若做成其轉殖鼠)則可以在 12 個月之後造成脂肪肝，17 個月之後形成肝癌。HCV 會促使肝 stellate 細胞活化,使 procollagen 1 增加,促進肝纖維化。免疫反應可以清除病毒,在 HIV 感染者,身體無法執行此免疫反應，HCV 感染進展快速。HCV 可以直接傷害肝臟,也可以經由免疫系統的 CTL,NK 及 NKT 細胞來執行肝傷害,或清除病毒。$\delta\gamma$ 細胞在肝多於周邊血液。CTL 經由 Fas/FasL 及 perforin,慢性感染的肝細胞會凋亡,造成肝傷害。NK 細胞可認知被感染的肝細胞,調節降低 MHC 之表現。可能有抗纖維化的作用。急性感染者,則 CTL CD38 被活化,IFN γ 分泌約 8 週左右回復了。

C 型肝炎的治療：

(1)干擾素(IFN)在使用的初期有快速的細胞死亡的效果,會增加 HCV(+)strain RNA,但減弱其蛋白的轉譯。後期則會活化 JNK 訊息通路。

(2)RIBAVIRIN 加 IFN：目前多用此，在第一基因型感染者有 45%左右沒效。

(3)Protease 抗抑制劑：以 NS3 protease 抑制劑 VX-950 為例,若加上 IFN,則病毒下降 5.5 log 10,若單獨使用，則也會下降 4log10,單獨使用 IFN 則下降 1log10。

(4)Polymerase 抑制劑

(5)Helicase 抑制劑

(6)Glycosidation 抑制劑:使病毒不能 asembly 及運出細胞。

4.脂肪肝傷害：

脂肪在肝細胞會增加其 Fas 受體之表現,增加其凋亡。若將 HepG2 細胞或培養的肝細胞,以飽合型脂肪酸共同作用,則會增進 caspase 相關之凋亡,這可以被 caspase 抑制劑所抑制。但不飽合脂肪酸則無此效果。

脂肪酸除了有細胞毒性，可以改變細胞死亡之機轉外，也會增加氧化壓力，造成粒線體功能不良；也會造成 ER 壓力，形成對胰島素之抗性,以增加細胞素的製造。

脂肪酸會使 cathepsin B 變不穩定，其釋放會活化 NFKB,及 TNF-α。飽含脂肪酸可以增進 JNK 通路(ERK),活化 Bax。總之脂肪酸可以引發 Fas 通路,使 Lysosome 不穩定,促成 TNFα 增加,促進脂肪凋亡,這與 JNK,caspase 通路有關。

脂肪肝炎的治療：

1) 必須合併飲食控制加上運動，才能增加對胰島素的敏感性，只有飲食或運動是不足的。飲食脂肪的型態，纖維攝取量等也會影響成果。

2) 減重手術

3) 藥物:原則上有下列幾類:(1)抗氧化壓力(2)以 SAMe 對抗 Glutathicne 缺乏(3)以 Aspirn 對抗發炎(4)以 caspase 抑制劑對抗細胞凋亡。例如：Pentoxifylline, Thalidomide, IMiDs, SelciDs, Rosiglitazone(PPAR-γ 之 ligand),Incretin, Vitamine E 等。

[註]：SAMe=S-adenosylmethionine Methionine +ATP $\xrightarrow{K+,Mg++}$ SAMe(~glutathione)

5.肝臟如何再生？

(1)肝再生的細胞及分子機轉與臨床應用：

肝細胞是主要的再生反應者，肝內的先驅細胞(Progenitor cell)或卵形細胞(oval cells)一般並不參與，只有在肝細胞無法再生時，才會負起再生的工作。這是很特別，也就是與幹細胞的生物學次序顛倒。在肝臟部分切除之後的再生，並不牽涉到肝幹細胞，在第一個月大約長兩倍體積。在活體肝移植捐肝者，一般再生至原來的 80%就停止了，並不會長到原來的 100%。

肝內網絡有(1)細胞素網絡，(2)生長因子網絡，(3)代謝網絡。細胞素網絡由 TNFα→TNFR1→NFKB→IL6→STAT3→細胞增生及存活 TNFα 可增進肝生長因子(HGF,TGFα,EGFα)引起肝細胞的增生。也可以促進代謝因子。

(2)肝幹細胞之臨床意義：

一般以肝細胞作移植，只<1%的植入細胞會長期存在或增生。但為了治療肝疾病，臨床上期望植入之細胞能佔有接受者之肝臟細胞的 90%。以 Tyrosinemia 模式之 Fah 基因剔除鼠為一很好的動物模式。若植入野生株老鼠的肝細胞，2 天後是單一細胞散在出現,20 天時停用 NTBC,會出現群聚細胞,50 天會佔有細胞 90%。以 Tyrosinemia 模式之 Fah 基因剔除鼠為一很好的動物模式。若植入野生株老鼠的肝細胞，2 天後是單一細胞散在出現，20 天時停用 NTBC，會出現群聚細胞，50 天會佔有細胞 90%。Fah -/-肝細胞 P21 被啟動，增加 17 倍。在部份肝切除之前，P21 已經增加了，它在 G1 至 S 的檢測點，在 P53 的下游 P21 增加，會造成 cell cycle 停止，所以雖作 2/3 肝切除，細胞也不增生。若作 Fah -/-及 P21 -/-的基因雙剔除鼠，則細胞會增生快速，而且只 6 星期就形成肝細胞癌了。所以失去 P21 易生肝癌。在膽汁滯留及 tyrosinemia 情況下，bid 之高度磷酸化，是造成細胞不死，也就是不凋亡的原因。膽道綁住造成肝細胞傷害，對 Fas 造成抗性對 acetaminophen 也造成抗性。P21 對於植入細胞之增生有很重要的角色。若接受捐贈者肝細胞的 cell cycle 停止，而捐贈者肝細胞不停止，則植入之細胞有生存增殖的優勢。未來肝臟受贈條件調整成適合植入細胞生存的狀況，例如利用放射線照射，化學物或藥物，抑制 Fah，或基因調整，可以達到此目標。

(3)由部分肝移植看肝再生：

一般正常肝只要 25%植入，即可存活，脂肪肝 30%則可活。約植入體重的 0.8-1%的肝重量可成功。植入較小的肝容易發生門脈高壓減低肝動脈的血流。給 Cardiotropin-1(IL-6 受體 gp130)，可增加再生及存活。給予 somatostation，FTY720，FK409，PGF1，或 Rapamycin 或作門脈下腔靜脈吻合術，或給予 TNF-α 抑制劑 pentoxify lline (PTX)，可以增進肝再生。PTX 經由增加 IL 6，可促進肝細胞再生。在 ILP 剔除鼠，給予 PTX 加 IL-6，會增加肝再生，若只給 PTX 但不加 IL-6，則不會存活。

血小板也與肝再生有關。如果去除血小板，則肝臟不易再生。這與 serotonin 之路徑有關。由 L-Tryptophon→5HT→5HT serotonin → 血小板之路徑若被阻斷，例如在 TPH 剔除小鼠則不生 serotonin，肝傷害後，也不會有肝再生；但若給予 serotonin，則會有肝再生。

6.基因與肝疾病

(1)肝纖維化

動物實驗常用，注射 ccl4，Thioacetamide，膽管綁住，或灌食毒物引發肝纖維化。Complement 5 也可增加 C 肝病人纖維化現象。反之若是給予 IL-6，IL-10，iNOS，plasminogen，IFN-γ，adiponectin，Telomerase，Bcl-XL 等，則可減少纖維化。活化肝臟 stellate 細胞(HSC)經由細胞內訊息路徑，會造成發炎及纖維化。活化 HSC，會刺激細胞外的間質(ECM)製造，且抑制 ECM 分解，TGFβ1 m RNA 會昇高。TLR 4 訊息增加也會增加肝纖維化，但若在 TLR4 剔除鼠，則綁膽管後肝纖維化會減少。

肝細胞損失後，會增加纖維化。若減少肝細胞損害，可以減少纖維化。這個過程中有 MIPZ 介入之，肝 kupffer cell 也介入此過程。改變基因，可以改變其纖維化的程度。

(2)核受體(NR)影響脂肪代謝：

NR 是基因調控最大的家族，在人體只有 48 個成員。它的基本構造有 NH2-A/B Region-DNA binding domain-ligand binding domain-cooH，48 個中有 5 個會受膽酸活化。

7.肝膽惡性腫瘤之機轉、診斷新方法及治療

(1)機轉：

腫瘤之特性是不需生長因子，會無限制的生長。下面幾個癌症訊息通路很重要，(a.)wnt/β-Latenin 路徑 (b)Receptor Tyrosine Kinase 路徑 (c)Sulfation SULF 1 Desulfates Heparan sulfate 若增加，可抑制腫瘤生長，若被抑制則會增加細胞生長，促進癌生長。(d)angiogenic 訊息傳遞：治療則可針對以上機轉，予以截斷其通路，作標靶治療。癌症幹細胞很可能存在於癌瘤中，可以產生腫瘤。

(2)診斷新方法：

a.新的腫瘤標記：

Glycoproteomics，用 Fucosylated GP73 偵測早期肝癌可有 92%敏感度，88%特異性。

b.新的影像檢查

Triple phase MRI, spiral CT, Dynamic MRI.

(3)治療：

手術，局部治療是基本療法，栓塞加化療藥物(緩慢釋放較佳)，以及化療用 Doxorubicin, 加 Cisplatinum,或用 Epirubicin, Mitoxantrone, 5FU, Paclitaxel, iridotecan, gemcitabin 等。

標靶治療針對下列訊息傳遞路徑：

- Wnt 路徑
- EGFR 路徑：Tyrosine Kinase inhibitor (TKI): Eriotinib, Gefitinib Sorafenib, Seocalcitol.
- Rof/MAPK 路徑: Sorafenib (VEGF-RAF 通路)

8.肝臟的本能免疫反應：

(1)肝臟如何認識病原菌：

本能的免疫系統會認識：”微生物非自己”Toll-Like Receptor(TLR)有至少 13 種，每一種經由相同或不同的訊息傳遞路徑，其中，LPS, Fibroneotin 及 RSV, MMTV 等病毒均經 TLR-4, 經由共同 adaptor Myd88 傳遞。TLR4-/-小鼠無法處理 Leishmania 等感染。

(2)TLR 使肝臟對肝傷害敏感化：

在各種肝傷害狀況或動物模式，例如酒精性肝炎，脂肪肝炎，D-galactosamine，缺血/再灌流等肝傷害，會在胆道細胞經由 TLRs,2,3,4,5 肝細胞經由 TLR1-9；stellate

細胞 TLRs 2,3,4,CD14;內皮細胞 TLR4, CD14; kupffer 細胞 TLR2,4,9, 引發敏感化,而造成肝傷害。將酒精餵給 TLR4 -/- 剔除鼠不會產生肝傷害，給 MyD88-/-剔除鼠，則仍會產生酒精性肝傷害。以抗生素可以降低內毒素(LPs)，減少肝傷害。TLR 的共同 adaptor, My D88 扮演肝傷害之重要角色。酒精經由 TLR4,CD14 造成肝傷害，食物中脂肪經由 TLR4-NFKB 造成肝傷害,在肝細胞受 C 肝病毒(HCV)感染之後 48 小時,IFN 相關之轉錄(transcription)增加,暫時活化 IRF-3,但接著逐漸下降，而 C 肝病毒之 protease 等蛋白製造也逐漸上昇。A 肝病毒會破壞 RIG-1 訊息傳遞系統及 MAVS/IPS-1 表現。

(3)TLR 與肝臟再生：再生的肝臟其訊息經由 TNFα→TNFR1→NFKB→IL6→STAT3。IL6 是由 Kupffer 細胞提供，而非肝細胞。在肝臟部份切除之後，若 IL-6 剔除鼠則再生不良而死亡。LPS 會啟動 TNFα 路徑,所以無菌環境下生長的小鼠，會降低肝再生能力。TLR4 突變也會造成對 LPS 無反應，而降低肝再生能力。若將 TLR2，TLR4，CD14，或 MyD88 基因剔除,則 SOCS-3,TNF,NFKB,IL-6,STAT3,SAA 在部分肝切除後均不反應了，影響肝再生能力。STAT3 連結 EGF 路徑，活化 MAPK 路徑。SOCS-3 是一個負向的調節者，使細胞素及 EGFR 訊息路徑及其他 TLR 相關路徑訊息減低。所以 SOCS-3 缺乏，會增加 STAT3 表現，造成肝細胞增生及癌形成。

III.與國際學者交換心得及學術交流：此次大會中許多知名學者與會，正好面對面與他們討論研究進展，設計及思考方向。

IV.參與 AASLD 大會節目評估委員會(Program Evaluation Committee)工作：這是本人第三年擔任本委員會工作，大會特地致贈感謝狀，感謝本人過去三年服務的貢獻。參加本委員讓我學習一個高水準的學術會議如何評估其學術節目內容及學術倫理,使未來節目做得更好。

V.參加 LiverInternation(SCI 雜誌)主編會議：本次會議是本人第四年參與主編工作的會議，目前本雜誌的投稿稿件一直在上昇，尤其本人負責的亞洲區。明年將是編輯委員第五年的工作，也是將與新主編及其團隊交接工作的時候了。自本編輯委員會在 2003 年接掌 Liver International , 將原來的雜誌 Liver 改名。由五大洲各一位主編合組主編群,由有副主編及編委多名。本人所負責的亞洲區成長最快速，全世界論文獲本刊接受刊載者以日本最多，其次是美國，及歐洲。台灣也有不錯的表現。中國投稿數量明顯逐年增加，值得注意。

三、考察參觀活動(無是項活動者省略)

四、建議

1. 像美國肝臟學會年會這樣高學術水準的會議應多鼓勵學者尤其年輕學者參加，並報告論文，以提昇我國研究在國際的可見度，並吸收新知，與國際學者多交流。
2. 參與國際學會的委員會，或國際雜誌的編輯工作，可以學習及提昇我國學術雜誌及學術會議的水準。

五、攜回資料名稱及內容

大會手冊(含論文摘要集)

六、其他

無。