行政院國家科學委員會補助專題研究計畫成果報告

B型肝炎病毒基因型與e抗原陽性孕婦在產後e抗原消失之關聯性

計畫類別：☑ 個別型計畫　　□ 整合型計畫

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計畫參與人員：

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一、中文摘要

目的：探討 B 型肝炎 e 抗原陽性孕婦在產後 e 抗原消失與 B 型肝炎基因型、病毒去氧核糖核酸（HBV DNA）濃度、與 precore/basal core promoter codon 型之關連性。

研究方法：從 2001 年 1 月至 2002 年 6 月止，共收集 40 例 B 型肝炎 e 抗原陽性孕婦為研究對象。在生產時及產後 3 至 4 個月分別採集孕婦血清，並追蹤至產後一年為止。全部血清分別檢查 HBeAg 價、HBV DNA 濃度、B 型肝炎基因型、以及 precore/basal core promoter codon 型，再進一步以 Mann-Whitney 及 Pearson chi-square tests 來檢討產後 e 抗原消失與上述 B 型肝炎病毒之變數作相關性分析。

結果：此 40 例 B 型肝炎 e 抗原陽性孕婦之平均年齡為 29.5± 5.3 歲，及平均分娩次數為 1.4± 0.7。在追蹤期間，5 例（A 組）在產後 3 至 4 個月內，其 e 抗原消失並持續至追蹤期間，剩下的 35 例（B 組）則在追蹤期間維持 e 抗原陽性。比較 A、B 兩組在生產前的 B 型肝炎病毒變數，發現與產婦平均年齡、生產次數、以及 HBV 的基因型分布，precore/basal core promoter codon 型等無統計學上的意義差。相對地，在 A 組之產婦其 HBeAg 價及 HBV DNA 濃度均比 B 組產婦低且具統計上意義差別，分別是 80 比 4,000（p=0.02）和<2.5 比 1,000（p=0.028）。

結論：此研究結果顯示 B 型肝炎 e 抗原陽性產婦，在生產後其 e 抗原消失與生產前其體內低 HBeAg 價及 HBV DNA 濃度有密切關連性，而與 HBV 基因型或 precore/basal core promoter codon 型無關。

〈關鍵詞〉：B 型肝炎病毒，e 抗原，帶原產婦，B 型肝炎病毒去氧核糖核酸，基因型

摘要

目的：

探討B型肝炎e抗原陽性孕婦在產後e抗原消失與B型肝炎基因型、病毒去氧核糖核酸（HBV DNA）濃度、與precore/basal core promoter codon型之關連性。

研究方法：

從2001年1月至2002年6月止，共收集40例B型肝炎e抗原陽性孕婦為研究對象。在生產時及產後3至4個月分別採集孕婦血清，並追蹤至產後一年為止。全部血清分別檢查HBeAg價、HBV DNA濃度、B型肝炎基因型、以及precore/basal core promoter codon型，再進一步以Mann-Whitney及Pearson chi-square tests來檢討產後e抗原消失與上述B型肝炎病毒之變數作相關性分析。

結果：

此40例B型肝炎e抗原陽性孕婦之平均年齡為29.5± 5.3歲，及平均分娩次數為1.4± 0.7。在追蹤期間，5例（A組）在產後3至4個月內，其e抗原消失並持續至追蹤期間，剩下的35例（B組）則在追蹤期間維持e抗原陽性。比較A、B兩組在生產前的B型肝炎病毒變數，發現與產婦平均年齡、生產次數、以及HBV的基因型分布，precore/basal core promoter codon型等無統計學上的意義差。相對地，在A組之產婦其HBeAg價及HBV DNA濃度均比B組產婦低且具統計上意義差別，分別是80比4,000（p=0.02）和<2.5比1,000（p=0.028）。

結論：

此研究結果顯示B型肝炎e抗原陽性產婦，在生產後其e抗原消失與生產前其體內低HBeAg價及HBV DNA濃度有密切關連性，而與HBV基因型或precore/basal core promoter codon型無關。

〈關鍵詞〉：

B型肝炎病毒，e抗原，帶原產婦， B型肝炎病毒去氧核糖核酸，基因型

Correlation of hepatitis B virus genotype, DNA level and precore/basal core promoter codon type with postpartum e clearance in HBeAg-positive carrier mothers

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<Running Title> Correlates of Postpartum e clearance

ABSTRACT

Objective: To correlate hepatitis B virus (HBV) genotype, HBV DNA level and precore/basal core promoter codon type with postpartum e clearance in HBeAg-positive carrier mothers.

Materials and Methods: From January 2001 to June 2002, a total of 40 consecutive HBeAg-positive mothers were enrolled. The blood samplings were taken at delivery and postpartum 3-4 months, and the follow-up period was until 1 year postpartum. All sera were tested by HBeAg, HBV DNA level, HBV genotype and precore/basal core promoter codon type. The correlation between HBV parameters and postpartum e clearance was analyzed by Mann-Whitney and Pearson chi-square tests.

Results: The mean age of the 40 HBeAg-positive carrier mothers was 29.9± 5.3 years with a mean parity of 1.4± 0.7. Five (group A) of them had postpartum e clearance and persisted thereafter, whereas the remaining 35 carrier mothers (group B) had persistent HBeAg-positivity during the follow-up period. The prepartum comparisons of HBV parameters between groups A and B showed that no statistical differences were noted among the mean values of age and parity, as well as prevalence in HBV genotype, precore/basal core promoter codon types. In contrast, carrier mothers in group A had lower HBeAg titer and HBV DNA level than those in group B (80 vs. 4,000, P=0.02, and <2.5 vs. 1,000 pg/ml, P=0.028, respectively).

Conclusion: Our data demonstrated that postpartum e clearance in HBeAg-positive carrier mothers intimately correlated with low HBeAg titer or HBV DNA level, instead of HBV genotype or precore/basal core promoter codon type.

<Key Words> Hepatitis B virus (HBV), HBeAg, carrier mothers, HBV DNA, genotype

INTRODUCTION

Taiwan is an endemic area of infection with hepatitis B virus (HBV) [1,2]. It has been well known that there are two ways to eradicate HBV infection- one is to prevent perinatal transmission [1-3], and the other is to prevent horizontal HBV transmissiom, such as screening of blood transfusion, antiviral drugs to induce HBeAg clearance or anti-e seroconversion in HBeAg-positive carriers, and education of people about the exact knowledge of HBV of HBV transmission, etc [3,4].

In our previous report, we find that some of HBeAg-positive carrier mothers can have e clearance or anti-e seroconversion postpartum [3]. However, we could only describe such a phenomenon then and we did not know what the exact mechanism is. In recent years, further studies show that HBV can be classified into genotypes of A – G [5-7], HBV genotype D is associated with more severe liver diseases than genotype A [8,9], as well as that genotype C has worse prognosis than genotype B [10-12]. Moreover, genotype D has more frequency in the mutations of precore/basal core promoter codon, and such mutations correlate well with HBeAg clearance or anti-e seroconversion as well as HBV DNA levels [13-19].

Thus, we propose a hypothesis that HBV genotype and precore/basal core promoter codon types may have intimate correlations with postpartum e clearance or anti-e seroconversion in HBeAg-positive carrier mothers after delivery. This study

was aimed to test such a hypothesis.

MATERIALS AND METHODS

Patients

Between January 2001 and June 2002, a total of 40 HBeAg-positive carrier mothers who visited our prenatal clinic at National Taiwan University Hospital and agreed to participate this study were consecutively enrolled. At delivery, maternal blood samples were taken and the second blood samplings for each carrier mother were taken within postpartum 3-4 months. The sera were stored at –70 ^{0}C until analysis.

Serologic tests

All sera were assayed for HBeAg by enzyme immunoassay (EIA) (Abbott Laboratories, North Chicago, IL, USA), and was also titrated by serial two- and ten-fold dilutions [3].

Quantitation of HBV DNA level

Serum HBV DNA was semiquantitated by an in-house slot hybridization assay, as previously described [10]. In brief, 50-ul of extracted HBV DNA, 100-ul of denatured solution, and 200-ul of neutralized solution were placed in the slotting apparatus, and vacuum-treated for 10min. The nylon membrane was then air-dried for 1h, and placed in a UV cross-linker for the fixation of DNA. After fixation, the nylon membrane was immersed in the hybridization buffer bag at 68 ^{0}C for 1h

(prehybridization), and the dilution series of control HBV DNA probes were then added to the bag overnight. The nylon membrane was then washed with 2x sodium saline citrate (SCC) and 0.1% sodium dodecylsulfate (SDS) at room temperature for 10min, and then with 0.1x SCC and 0.1% SDS at 68 ^{0}C for 20 min. Finally, the membrane was subjected to detection with a digoxigenin detection kit (DIG DNA Labeling and Detection Kit; Boehringer Mannheim, Mannheim, Germany), according to the manufacturer's instructions. The range of HBV DNA concentration was determined after comparison with the band intensities of serially diluted control probes. The detection limit of our in-house slot hybridization assay was 2.5 pg/ml, as determined by serial dilutions of HBV plasmid with known concentrations.

Genotyping of HBV

The identification of HBV genotypes was performed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of the surface gene of HBV, as previously described [10]. Briefly, DNA was extracted from 200-ul serum samples, using the QIAmp blood kit (Qiagen, Chatsworth, CA, USA), and the fragment of the HBV genome between nucleotide position 256 and 796 was then amplified. The PCR products were subsequently treated with restriction enzymes. After incubation, the samples were run on a 3% agarose gel and stained with ethidium bromide. Six genotypes (A-F) of HBV could be identified by the restriction patterns

of the DNA fragments. An unclassified genotype was defined as one with an unpredicted or atypical restriction pattern. To avoid false-positive results, instructions to prevent cross-contamination were strictly followed, and results were considered valid only when they were obtained in duplicate.

Amplification and sequencing of precore/basal core promoter gene

For the first-stage PCR, 25-ul of reaction mixture, containing 2-ul of the DNA sample, 1x PCR buffer (10 mM Tris-HCl, pH 9.0, 50 mM 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, and 1 unit of Taq DNA polymerase was amplified in a thermal cycler (Perkin-Elmer Cetus, Norwalk, CT, USA) for 35 cycles, as previously described [20]. Each cycle entailed denaturation at 95 ^{0}C for 60s, primer annealing at 52 ^{0}C for 30s, and extension at 72 ^{0}C for 60s, with a final extension step at 72 ^{0}C for 10min. After the first amplification, 1-ul of the PCR products was reamplified for another 35 cycles with 100 ng of each inner primer. For the precore region, the outer sense primer was 5'-CTGGGAGGAGTTGGGGGA-3', nucleotide position 1730-1747; the outer antisense primer was 5'-CAATGCTCAGGAGACTCTAAGGC-3', nucleotide positions 2043-2021; the inner sense primer was 5'-GGTCTTTGTACTCGGAGGCTG-3', nucleotide position 1763-1783; and the inner antisense primer was 5'-GTCAGAAGGCAAAAAAGAGAG-3', nucleotide

position 1966-1946. For the basal core promoter region, the outer sense primer was 5'-CTAGCCGCTTGTTTTGCTCG-3' nucleotide positions 1282-1301; the outer antisense primer was 5'-CACAGCTTGGAGGCTTGAAC-3', nucleotide positions 1881-1862; the inner sense primer was 5'-CTCATCTGCCGGACCGTGTG-3', nucleotide positions 1562-1581; and the inner antisense primer was 5'-TAGGACATGAACAAGAGATG-3', nucleotide positions 1859-1840. Nucleotide sequences of the amplified products were directly determined by using fluorescence-labeled primers with a 377 Automatic Sequencer (Applied Biosystems, Foster City, CA, USA). Sequencing conditions were specified in the protocol for Taq DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems). The inner primers were used as sequencing primers for both directions of each region.

Statistical analysis

Data values were analyzed by Mann-Whitney test, or Pearson chi-square test when appropriate. All of the tests of significance were two-tailed, and a P value of <0.05 was considered statistically significant.

RESULTS

The mean age of these 40 HBeAg-positive carrier mothers was 29.9 ±5.3 years with a mean parity of 1.4± 0.7. Within 3-4 months after delivery, 5 (group A) of them had postpartum e clearance and they persisted during 1-year follow-up, whereas the remaining 35 carrier mothers (Group B) had persistent HBeAg-positivity postpartum during the follow-up period. The prepartum comparisons of HBV parameters between groups A and B are shown in Table 1. No statistical differences were noted concerning the mean values of age and parity, as well as prevalence in HBV genotypes, precore stop codon types and basal core promoter codon types between groups A and B. No mutant types of precore stop codon (G1896A) or basal core promoter codon (A1762T and G1764A) were noted in these 40 HBeAg-positive carrier mothers. In contrast, carrier mothers in group A had lower HBeAg titer and HBV DNA level than those in group B (80 vs. 4,000, P=0.02, and <2.5 vs. 1,000 pg/ml, p=0.028, respectively).

Table 2 shows the postpartum comparisons of HBV parameters between groups A and B. No significant differences in prevalence of HBV genotypes and precore stop codon type were noted between groups A and B. However, the prevalence of basal core promoter codon type showed less mixed type in group A carrier mothers than those in group B cases after delivery (P=0.019). Besides, all the 5 carrier mothers in

group A had postpartum e clearance and they had undetectable HBV DNA level (<2.5 pg/ml) which were significantly lower than those in group B cases (P=0.002)

Concerning the prepartum predictors of postpartum e clearance in HBeAg-positive carrier mothers, we found that low HBeAg titer (<650) and HBV DNA level (<20 pg/ml) showed the positive prediction value (PPV) of 80% and 80%, while the negative prediction values (NPV) of 74% and 83%, respectively.

DISCUSSION

In our previous study, we reported that the immunosuppression status during pregnancy was removed and rebound of immune response occurred after delivery. Furthermore, the effect of pregnancy and delivery on the immune status only lasted within postpartum 3-4 months [3]. Thus, we compared the blood samplings for HBV parameters between at delivery and postpartum 3-4 months to evaluate the effect of pregnancy and delivery on the HBV parameters in HBeAg-positive carrier mothers.

In the present study, we found that 5 (12.5%) out of the 40 HBeAg-positive carrier mothers had postpartum e clearance, and this figure is similar to that of 16.6% (5/30) in our previous report [3]. Our data further demonstrated that the prepartum predictors of e clearance after delivery are not correlated with age, parity, HBV genotype, precore stop codon and basal core promoter codon. In contrast, the low HBeAg titer (<650) and HBV DNA level (<20 pg/ml) are well correlated with postpartum e clearance in HBeAg-positive carrier mothers. These findings are consistent with our previous report [3]. Thus, we may infer that the lower prepartum HBV level in HBeAg-positive carrier mothers, the more postpartum e clearance occurs.

It was also noted that none of mutant types of either precore stop codon or basal core promoter codon existed among these HBeAg-positive carrier mothers. This

finding is rather consistent to the previous reports [16,18-20], which showed that more prevalence rates in mutant types of either precore stop codon or basal core promoter codon existed in HBeAg-negative patients rather than in HBeAg-positive cases [16,18-20]. Furthermore, it is noteworthy that the HBV DNA level was extremely low (less than the sensitivity of the test method, <2.5 pg/ml) in HBeAg-negative patients such as group A carrier mothers after delivery (Table 2). This means that HBeAg-negative carrier mothers almost have low HBV DNA level. In contrast, not all of HBeAg-positive carrier mothers have high HBV DNA level as shown in Table 1, namely, some of them may have low HBV DNA level.

In conclusion, our data demonstrated that postpartum e clearance in HBeAg-positive carrier mothers intimately correlated with low HBeAg titer or HBV DNA level, instead of HBV genotype, or precore/basal core promoter codon type.

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Table 1. Prepartum comparisons of HBV parameters between HBeAg-positive carrier mothers with (Group A) and without postpartum e clearance

HBV	A (n=5)	B (n=35)	P
Age (years)	28.0 (24.5-33.0)	29.0 (27.0-34.0)	0.696#
Parity	1 (1-2)	1 (1-2)	0.107#
HBeAg titer	80 (4-2040)	4000 (2000-10000)	0.02#
DNA (pg/ml)	<2.5 (0-419)	1000 (20-2500)	0.028#
Genotype			0.198*
B	5	26	
C	0	9	
Precore stop codon			0.449*
mixed type	2	6	
wild type	3	27	
not available	0	2	
Basal core promoter codon			0.145*
mix type	0	3	
wild type	2	25	
not available	3	7	

#: by Mann-Whitney test; *: by Pearson chi-square test; ( ): 25%-75% intraquantile range.

Table 2. Postpartum comparisons of HBV parameters between HBeAg-positive carrier mothers with (group A) and without (group B) postpartum e clearance

HBV	A (n=5)	B (n=35)	P
DNA (pg/ml)	<2.5	1800 (200-3100)	0.002#
Genotype			
B	4	26	0.783*
C	1	9	
Precore stop codon			0.076*
mix type	1	3	
wild type	1	25	
not available	3	7	
Basal core promoter codon			0.019*
mix type	0	4	
wild type	1	24	
not available	4	7	

#: by Mann-Whitney test; *: by Pearson chi-square test; ( ): 25%-75% intraquantile range.

Table 3. Prepartum prediction values of HBeAg titer and HBV DNA between HBeAg-positive carrier mothers with (group A) and without postpartum e clearance

HBV	A (n=5)	B (n=35)	P#
HBeAg titer*			0.002
<650	4	6	
≧650	1	29	
DNA (pg/ml)**			0.015
<20	4	9	
≧20	1	26	

#: by Pearson chi-square test;

*: The cut-off value shows the positive prediction value (PPV) of 80% and negative prediction value (NPV) of 74%.

**: The cut-off value shows the positive prediction value (PPV) of 80% and negative prediction value (NPV) of 83%.