Influential Factors of Hepatitis B Virus cccDNA in Peripheral Blood Mononuclear Cells Among HBsAg-Positive Pregnant Females Neonates

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Abstract

Background: Although many studies have measured HBV cccDNA molecules in Peripheral Blood Mononuclear Cells (PBMC) from patients with active chronic hepatitis B, the current pilot study found PBMC HBV cccDNA in PBMC among HBsAg-positive mothers and their neonates. However, the risk factor of HBV cccDNA in PBMC among HBsAg-positive pregnant female's neonates remains unclear.

Objectives: The aim of this study was to explore influential factors of HBV cccDNA in PBMC among HBsAg-positive pregnant female's neonates.

Methods: Peripheral blood samples and clinical data were collected from 151 pregnant females, who were positive for hepatitis B surface antigen (HBsAg) in the Third People Hospital of Taiyuan City. Blood samples from 152 neonates were collected before immune prophylaxes administration and tested for HBV markers, HBV DNA in serum, and HBV DNA in PBMC. Bayesian logistic regression with Cauchy prior were used to measure the association between maternal characteristics, neonatal characteristics, and HBV cccDNA in PBMC of neonates.

Results: Among neonates of HBsAg-positive mothers, the positive rate of cccDNA in PBMC was 4.61% (7/152). Maternal PBMC HBV cccDNA positivity (OR = 18.411, 95%CI: 3.025 - 66.022) and neonates PBMC rcDNA positivity (OR = 13.529, 95% CI: 1.948 - 93.690) were associated with HBV cccDNA in neonatal PBMC, respectively.

Conclusions: The study suggested that HBV cccDNA can be detected in PBMC of HBsAg-positive mother's neonates. Maternal PBMC HBV cccDNA positivity and neonatal PBMC rcDNA positivity are risk factors of HBV cccDNA in PBMC of neonates.

Keywords: PBMC, HBV, HBV cccDNA, Influential Factor

1. Background

Hepatitis B virus (HBV) infection, a major public health problem worldwide, increases the risk of terminal liver disease in more than 250 million people (1). China is in the intermediate prevalence region of HBV (2). Vertical transmission of hepatitis B virus is a major reason for the spread of HBV in areas where it is prevalent (3). Hepatitis B virus transmission from the mother to infant includes intrauterine transmission, intrapartum transmission, and puerperal transmission. Cellular transmission by peripheral blood mononuclear cells (PBMC) is regarded as a possible route for HBV intrauterine transmission. Some studies have shown that maternal HBV can traverse the placenta eventually by sera and PBMC and then may lead to HBV intrauterine transmission (4-7). Hepatitis B virus intrauterine transmission was defined as finding HBsAg and/or HBV DNA positivity in the peripheral blood of neonates within 24 hours of birth and before active or passive immune prophylaxis (8, 9). Previous researches showed that the sensitivity and accuracy of HBV covalently-closed circular DNA (cccDNA) was better than that of serum HBV DNA, which was widely regarded as the most specific biomarker of hepatitis B virus replication (10). Hepatitis B virus infections are maintained by the presence of a small and regulated number of episomal viral genome cccDNA in the nuclei of infected cells. Hepatitis B virus cccDNA is the template for the replication of HBV, which plays a key role in viral infection and persistence (11, 12).

Many studies have only measured HBV cccDNA molecules in PBMC from patients with active chronic
hepatitis B (13), while the current research group found HBV cccDNA in PBMC among HBsAg-positive mothers and their neonates (14). The risk factors of HBV cccDNA in PBMC among HBsAg-positive pregnant female’s neonates remains unclear.

2. Objectives

The aim of this study was to explore the influential factors of HBV cccDNA in neonatal PBMC and to provide a theoretical basis for exploring potential etiology of HBV intrauterine transmission.

3. Methods

Eligible study subjects were HBsAg-positive mothers, who had given birth in the third people hospital of Taiyuan city between 1st of June 2001 and 31st of December 2002. A total of 151 pregnant females were eligible for the study. The basic information, including maternal demographics, history of disease, and HBV infection details before and during pregnancy, of the HBsAg-positive mothers and neonates were collected by well-trained interviewers utilizing standardized and unified questionnaires by face-to-face interviews or medical records. The research protocol was approved by the ethics committees of Shanxi Medical University (No:2016LL143), and all mothers signed a written informed consent.

3.1. Sample Collection

All participants donated 5 mL of peripheral blood before delivery, and 5 mL of femoral venous blood was collected from each infant within 24 hours after birth prior to inoculations of hepatitis B vaccine and HBig. Blood samples were processed within 24 hours of being drawn. Peripheral blood mononuclear cells were isolated by Ficoll-Paque density gradient centrifugation. The plasma samples and PBMC were stored at -80°C for further experiments.

3.2. Serological Tests

HBsAg and HBeAg were measured by the enzyme-linked immunosorbent assay (ELISA) (Shanghai Kehua Biotechnology, Shanghai, China). All procedures were performed according to the manufacturers’ instructions.

3.3. Molecular Tests

The total DNA from plasma or PBMC was extracted with hydroxybenzene-chloroform-isoomyl alcohol and the integrity of DNA was assessed by gel electrophoresis. The blood plasma or PBMC, which was positive for HBsAg, HBeAg, and HBV DNA was selected as the positive control, and the blood plasma or PBMC, which was negative for HBsAg, HBeAg, and HBV DNA was selected as the negative control. Additionally, sterile water was used as the blank control. All the three control specimens and case specimens were used for DNA isolation and PCR.

3.3.1. Hepatitis B Virus DNA Tests

Hepatitis B virus DNA was tested by nested Polymerase Chain Reaction (n-PCR) using nested primers (15) (Table 1). The first round of nested PCR was performed in 50-µL reaction system, which contained 25 µL of template, 5 µL 10 × Buffer, 4 µL MgCl₂ (25 mM each), 1 µL primer (50 pM/µL), 1 µL dNTP mixture (10 mM each), and 0.4 µL Taq DNA polymerase (5 units/µL). Thermal cycle parameters included pre-denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 50 seconds, annealing at 55°C for 50 seconds, extension at 72°C for 5 minutes, and finally extension for 10 minutes at 72°C. The first round product of 5 µL was taken as a template for the second round of amplification with the same components and parameters as the first round of PCR. The PCR amplification products were electrophoresed on 2% agarose gels stained with ethidium bromide and examined under UV light.

3.3.2. Hepatitis B Virus cccDNA Tests

Hepatitis B virus rcDNA and cccDNA in PBMC were tested by selected polymerase chain reaction (s-PCR) using selected primers (16) (Table 1). The PCR was performed in a 20-µL reaction system, which contained 15 µL of template, 2 µL 10 × Buffer, 0.15 µL MgCl₂ (25 mM each), 0.2 µL primer (50 pM/µL), 0.4 µL dNTP mixture (10 mM each), and 0.2 µL Taq DNA polymerase (5 units/µL). Thermal cycle parameters, included pre-denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 60 seconds, annealing and extension at 72°C for 3 minutes, and finally extension for 5 minutes at 72°C. The PCR amplification products were electrophoresed on 2% agarose gels stained with ethidium bromide and examined under UV light. Then, the products were sent for sequencing and blasted with HBV standard sequences.

3.4. Statistical Analysis

Neonates, for whom HBV cccDNA in PBMC was positive, were selected as cases, and those, for whom HBV cccDNA in PBMC was negative, were selected as controls. In univariate analyses, chi-square test was used for categorical data and Student’s t-test was used for continuous variables. These analyses were performed using SPSS version 19.0 software. Bayesian logistic regression with Cauchy was used prior to estimation of odds ratio (OR) and 95% confidence interval (CI). The analyses were performed using R version 3.2.2 software. P < 0.05 was considered statistically significant.
Table 1. The Specific Primers Used for Amplifying Hepatitis B Virus DNA

| Amplifying Fragment | Primer Name | Sequences (5’ - 3’) | Position, nt | Product Size, bp |
|---------------------|-------------|----------------------|--------------|-----------------|
| HBV DNA, 1st round  | Forward     | CTGCCGTGGTGGCTCCAGTT | 59 - 76     | 699             |
|                     | Reverse     | CAATTACACATCATCCA     | 758 - 741   |                 |
| HBV DNA, 2nd round  | Forward     | CCTGCTCGTGTTACAGGC   | 189 - 206   | 500             |
|                     | Reverse     | GGGCTAGTAACTGAGG      | 689 - 672   |                 |
| HBV, rcDNA          | Forward     | CGAACCGGGGGCAGCTCTTTGAG | 1515 - 1542 | 243             |
|                     | Reverse     | CTAATCCTCTCCCCAGCTCTCCCAGT | 1758 - 1731 |                 |
| HBV cccDNA          | Forward     | CGAACCGGGGGCCAGCTCTTTGAG | 1515 - 1542 | 373             |
|                     | Reverse     | CAAGGCACAGCTGGTGGCTCAAGT | 1888 - 1861 |                 |

Table 2. Distributions of Maternal and Neonatal Characteristics in Cases and Controls

| Characteristics                  | Cases, (N = 7), No. (%) | Controls, (N = 145), No. (%) | P Value |
|----------------------------------|-------------------------|------------------------------|---------|
| Maternal characteristics         |                         |                              |         |
| Age, y, mean (SD)                | 28.30 (4.13)            | 29.00 (7.65)                 | 0.679   |
| Highest educational levels       |                         |                              | 0.699   |
| < High school                    | 5 (71.4)                | 80 (57.1)                    |         |
| ≥ High School                    | 2 (28.6)                | 60 (42.9)                    |         |
| Gestational weeks                |                         |                              | 0.582   |
| < 37                             | 0 (0.0)                 | 12 (8.3)                     |         |
| 37 - 41                          | 6 (85.7)                | 123 (84.8)                   |         |
| ≥ 41                             | 1 (14.3)                | 10 (6.9)                     |         |
| Neonates characteristics         |                         |                              | 0.442   |
| Gender                           |                         |                              |         |
| Girl                             | 2 (28.6)                | 72 (51.1)                    |         |
| Boy                              | 5 (71.4)                | 69 (48.9)                    |         |
| Deformity                        |                         |                              | 1.000   |
| Yes                              | 0 (0.00)                | 2 (1.5)                      |         |
| No                               | 7 (100.0)               | 134 (98.5)                   |         |
| Weight, mean (SD)                | 3207.1 (333.4)          | 3300.0 (449.4)               | 0.591   |
| Height, mean (SD)                | 49.7 (1.49)             | 49.5 (1.64)                  | 0.769   |

4. Results

4.1. Result of Molecular Tests

There were 151 HBsAg-positive mothers and 152 neonates, which included one pair of twins. In all of the neonates, ten neonates had positive results for HBsAg and 5 neonates had positive results for HBV DNA in serum. Two neonates were infected in the form of occult infection. Hepatitis B virus intrauterine transmission was defined as finding HBsAg and/or HBV DNA positivity in the peripheral blood of neonates within 24 hours of birth and before active or passive immune prophylaxis. The rate of HBV intrauterine transmission was 7.9% (12/151). In all of neonates, 7 neonates had positive results for HBV rcDNA and 35 neonates had positive results for HBV cccDNA in PBMC.

4.2. Associations Between Maternal Characteristics, Neonatal Characteristics, and Hepatitis B Virus cccDNA in Peripheral Blood Mononuclear Cells of Neonates

Neonates, for whom HBV cccDNA in PBMC was positive were selected as cases, and those, for whom HBV cccDNA in PBMC was negative, were selected as controls. The demographic characteristics were the same between cases and controls (Table 2). In univariate analyses (Table 3), maternal PBMC HBV rcDNA positivity (P = 0.009), maternal PBMC HBV cccDNA positivity (P < 0.001), neonatal HBsAg positivity (P = 0.007), and neonatal PBMC rcDNA positivity (P < 0.001) were significantly associated with HBV cccDNA in neonatal PBMC.

After adjusting for related covariates (Table 4), maternal PBMC HBV cccDNA positivity increased the risk of HBV replication in neonatal PBMC (OR = 18.411, 95% CI: 3.025 - 66.022). Neonatal PBMC rcDNA positivity was significantly associated with HBV replication in neonatal PBMC (OR = 13.529, 95% CI: 1.948 to 93.690).

5. Discussion

In this study, 7.9% of neonates born to HBsAg-positive mothers showed HBV intrauterine transmission, which was within the range of 5% to 40% in China (17, 18).

After uncoating, HBV was transported to the nucleus, in which the virus would be converted to a covalently
Table 3. Univariate Analyses of Associations Between Maternal Characteristics, Neonate’s Characteristics, and HBV Replication in PBMC of Neonates

| Characteristics                        | Cases   | Controls | P Value |
|----------------------------------------|---------|----------|---------|
|                                        | No | Yes | No | Yes |
| Pregnancy                              |    |     |    |     |
| History of hepatitis                   | 6 (85.7) | 1 (14.3) | 117 (81.2) | 27 (18.8) | 1.000 |
| Family history of HBV infection        | 4 (57.1) | 3 (42.9) | 93 (68.4) | 43 (31.6) | 0.681 |
| History of blood transfusion           | 6 (85.7) | 1 (14.3) | 140 (97.9) | 3 (2.1) | 0.276 |
| History of acupuncture                 | 6 (100.0) | 0 (0.0) | 134 (93.1) | 10 (6.9) | 1.000 |
| History of dental treatment            | 5 (83.3) | 1 (16.7) | 105 (72.9) | 39 (27.1) | 0.794 |
| HBV vaccine injection                  | 6 (100.0) | 0 (0.0) | 130 (90.9) | 13 (9.1) | 1.000 |
| History of abortion or induced labor   | 7 (100.0) | 0 (0.0) | 93 (64.6) | 51 (35.4) | 0.096 |
| History of labor                       | 5 (71.4) | 2 (28.6) | 112 (77.8) | 32 (22.2) | 0.655 |
| Pregnancy                              |    |     |    |     |
| Medication use during pregnancy        | 6 (100.0) | 0 (0.0) | 108 (75.0) | 36 (25.0) | 0.336 |
| Antepartum hemorrhage                  | 6 (100.0) | 0 (0.0) | 130 (90.3) | 14 (9.7) | 1.000 |
| Threatened premature labor             | 6 (100.0) | 0 (0.0) | 143 (99.3) | 1 (0.7) | 1.000 |
| HBIG injection                         | 4 (57.1) | 2 (42.9) | 44 (30.0) | 98 (69.0) | 0.087 |
| Maternal HBeAg positive                | 3 (42.9) | 4 (57.1) | 90 (62.4) | 55 (37.6) | 0.431 |
| Maternal serum HBV DNA positive        | 4 (57.1) | 3 (42.9) | 80 (55.2) | 65 (44.8) | 1.000 |
| Maternal PBMC HBV rcDNA positive       | 1 (14.3) | 6 (85.7) | 96 (66.2) | 49 (33.8) | 0.009 |
| Maternal PBMC HBV cccDNA positive      | 2 (28.6) | 5 (71.4) | 135 (93.1) | 10 (6.9) | < 0.001 |

Delivery

| Variables               | β    | S.E.  | Z     | P Value | OR   | OR 95% CI |
|-------------------------|------|-------|-------|---------|------|-----------|
| Caesarean section       | 3 (57.1) | 4 (42.9) | 83 (59.3) | 57 (40.7) | 1.000 |
| After delivery          |    |     |    |     |
| Neonates HBsAg positive | 4 (57.1) | 3 (42.9) | 133 (94.6) | 7 (5.4) | 0.007 |
| Neonates anti-HBs positive | 6 (100.0) | 0 (0.0) | 117 (97.3) | 3 (2.7) | 1.000 |
| Neonates HBeAg positive | 4 (57.1) | 3 (42.9) | 92 (66.7) | 46 (33.3) | 0.689 |
| Neonates anti-HBe positive | 4 (66.7) | 2 (33.3) | 71 (59.2) | 49 (40.8) | 1.000 |
| Neonates HBcAg positive | 6 (100.0) | 0 (0.0) | 119 (99.2) | 1 (0.8) | 1.000 |
| Neonates anti-HBc positive | 3 (50.0) | 3 (50.0) | 26 (21.7) | 94 (78.3) | 0.134 |
| Neonates serum HBV DNA positive | 6 (85.7) | 1 (14.3) | 129 (97.0) | 4 (3.0) | 0.229 |
| Neonates PBMC HBV rcDNA positive | 1 (14.3) | 6 (85.7) | 116 (80.0) | 29 (20.0) | <0.001 |

*Values are expressed as No. (%).

Table 4. Multivariate Analysis of Associations Between Maternal Characteristics, Neonates Characteristics and Hepatitis B Virus Replication in Peripheral Blood Mononuclear Cells of Neonates

| Variables              | β    | S.E.  | Z     | P Value | OR   | OR 95% CI |
|------------------------|------|-------|-------|---------|------|-----------|
| Intercept              | -5.754 | 1.205 | -4.772 | 1.82E-06 | -    | -         |
| Maternal PBMC HBV cccDNA | 2.913 | 0.921 | 3.162 | 1.000 | 18.411 | 3.025 - 66.022 |
| Neonates PBMC HBV rcDNA | 2.604 | 0.988 | 2.635 | 0.008 | 13.529 | 1.948 - 93.690 |
| Maternal PBMC HBV rcDNA | 0.819 | 0.996 | 0.822 | 0.411 | 2.269 | 0.321 - 16.034 |
| Neonates HBsAg         | -0.005 | 0.241 | -0.223 | 0.981 | 0.994 | 0.618 - 1.595 |

closed circular molecule (cccDNA), which would serve as the template for transcription (19). Although HBV is generally considered to be hepatotropic, HBV specific nucleic acids and relative antigen can be detected in many extra-
hepatic tissues (20, 21), and it has been proved that PBMC may be the extrahaepatic place of HBV transcription and translation (22, 23). In the present study, 15 mothers and 7 neonates were positive for HBV cccDNA in PBMC, which suggested that HBV can exist and be replicated in PBMC.

Besides, it was observed that an increased risk of HBV cccDNA in PBMC of neonates was associated with maternal PBMC HBV cccDNA positivity. The positivity of HBV cccDNA is a sign of HBV replication (2, 24). Hepatitis B virus cccDNA in neonatal PBMC may suggest that there may be HBV replication in neonates. However, HBV cccDNA in neonatal PBMC may come from maternal PBMC. Furthermore, PBMCs are immigrant cells from mother to neonate’s blood stream (25), which can carry cccDNA into neonates. Otherwise, they may be due to blood transfec-

tion of neonates with the mother during delivery. The source of neonatal PBMC HBV cccDNA remains to be explored further. However, the persistence of HBV cccDNA is the main source of failure to eliminate HBV, which can cause serious outcomes (26). Engineered site-specific nucleases and RNA interference therapeutics could clear or silence cccDNA (27). Thus, PBMC HBV cccDNA in HBsAg positive pregnant females can be detected during the gestation period, and clearance of HBV cccDNA by anti-viral treat-

tment to decrease the risk of HBV replication in neonatal PBMC could be achieved.

Neonatal PBMC HBV rcDNA positivity may influence its PBMC HBV cccDNA. Hepatitis B virus is a small DNA virus that replicates by protein-primed reverse transcription (28). After infection, HBV rcDNA is converted to cccDNA, which serves as a viral persistence reservoir. This suggests that the development of HBV infection may be controlled by preventing transformation from rcDNA to cccDNA.

The researchers firstly detected HBV cccDNA in PBMC of HBsAg-positive pregnant females and their neonates. Furthermore, this study firstly focused on the risk factors of HBV cccDNA in PBMC among HBsAg-positive pregnant female’s neonates. The current study has laid the foundation of subsequent research. However, the source of HBV cccDNA in neonatal PBMC is still unclear. The neonates, who were positive for HBV cccDNA in PBMC were not followed up and thus the persistence of HBV cccDNA in neonatal PBMC could not be understood. Future research will follow up neonate, who was positive for HBV cccDNA in PBMC and observe the nucleotide divergence of HBV cccDNA in mater-

nal PBMC and neonatal PBMC and the variation of HBV sequences.

5.1. Conclusion

Hepatitis B virus cccDNA could be detected in PBMC among neonates born to HBsAg-positive mothers, and the positive rate of HBV cccDNA in PBMC was 4.61%. Maternal PBMC HBV cccDNA positivity and neonates PBMC rcDNA positivity may increase the risk of HBV cccDNA positivity in PBMC of neonates. This preliminary conclusion will provide the basis for future research of HBV intrauterine infection.

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Footnotes

Conflict of Interest: None declared. Completed disclosure of interest form is available online as supporting in-
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