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Research paper

Epigenome-wide study for the offspring exposed to maternal HBV infection during pregnancy, a pilot study

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ABSTRACT

Background & aim: Hepatitis B virus (HBV) can be transmitted to infants, and is related to infants' later disease risk. Epigenetic change (such as DNA methylation) may be mechanism underlying the relationship. In this study, we aimed to investigate whether prenatal HBV infection could alter DNA methylation status in newborns.
Method: We selected 12 neonates with intrauterine HBV infection whose mothers were HBsAg-positive during pregnancy, relative to 12 HBV-free neonates with HBsAg-negative mothers. The pattern of genome-wide DNA methylation in the umbilical cord blood was investigated by Illumina Infinium Human Methylation 450K BeadChip.
Result: The average level of global methylation in infected neonates exposed to maternal HBV infection was not significantly different from controls. However, after adjusting for multiple comparisons, we found differential significance in the cases group compared to the controls for 663 CpG sites, associated with 534 genes. Among these sites, 53.85% (357/663) had decreased methylation ($\Delta M < 0$) and 46.15% (306/663) had increased methylation ($\Delta M > 0$). The average percentage change ($\Delta\beta$) in methylation ranged from −46% to 36%. Validated by pyrosequencing, we identified 4 significantly differentially methylated CpG sites in the *KLHL35* gene and additional CpGs for the *CPT1B* gene. These genes play a role in the development of hepatocellular and colorectal carcinoma and fatty acid oxidation, suggesting the candidature of these genes in HBV related disease.
Conclusion: Prenatal HBV exposure, even without malformation or preterm birth, may alter the epigenome profile in newborns. We identified a set of genes with differentially methylated CpG sites presented in the cord blood of HBV-infected newborns with HBsAg-positive mothers, demonstrating that DNA methylation status at birth can be used as a biomarker of prenatal exposure. These DNA methylation differences suggest a possible role for epigenetic processes in neonatal development in response to prenatal HBV exposure.

1. Introduction

According to the report of World Health Organization (WHO), there are about 2 billion people infected with hepatitis B virus (HBV) among the 6 billion human beings around the world (Dienstag and Hepatitis, 2008). China has a relatively severe HBV epidemic situation; it has been reported that 33% of the people who had positive HBsAg in the world were from China, with 37% of the HBV-related death (Wang et al., 2004; Liang, 2010). HBV infection can induce acute hepatitis B, chronic hepatitis B (CHB), liver cirrhosis (LC), and hepatocellular carcinoma (HCC), and thus remains a threat to people's health and life. The virus of hepatitis B can infect the fetus via transmitting from maternal peripheral blood to cord blood through placentas. The high load of maternal hepatitis B e antigens (HBeAg) or HBV-DNA is one of the risk factors for intrauterine infection of newborns (Lanfang et al., 2014). Research showed that infants with HBV-loading mother were more likely to be subjected to brachial plexus injury (Salemi et al., 2014). However, the mechanism governing the relationship of maternal HBV infection and its effect on newborns remains unclear.

Epigenetic changes may provide ideas to explore this relationship. During pregnancy, mothers and newborns are vulnerable to many kinds of stressors. The impact of maternal exposure to environmental factors

Abbreviation: BMI, Body mass index; HBsAg, Hepatitis B Surface Antigen; HBxAg, Hepatitis B x antigens; RUV, Removal Unwanted Variation

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on the methylation status of fetus has been studied in recent years. Evidence suggested that maternal smoking during pregnancy may lead to obesity and elevated blood pressure in children (Brion et al., 2008; Cupul-Uicab et al., 2012). Meanwhile, it has been reported that children related to maternal smoking have a set of genes with methylation changes presenting at birth (Joubert et al., 2012; Küpers et al., 2015; Ivorra et al., 2015; Markunas et al., 2014). The connection of prenatal micronutrient levels, placental epigenetic status and birth weight was suggested in Maccani's study (Maccani et al., 2015).

Previous researches have shown that HBV may has the ability to change DNA methylation status of host genes via activating DNA methyltransferase expression by hepatitis B x protein (Lee et al., 2005; Park et al., 2007; Zheng et al., 2009; Jung et al., 2007), which accelerates the process of relative diseases (Yang et al., 2003; Tchou et al., 2000; Brechot et al., 2000). In the tumor tissues and peripheral blood samples of HBV-related HCC patients, it was obvious to find that promoters of tumor suppressor genes, such as *RASSF1A*, *p16*, *GSTP1*, were hypermethylated (Saelee et al., 2010; Zhang et al., 2002; Zhong et al., 2003; Zhong et al., 2002). Decrease in mRNA expression was related to hypermethylation of *E-cadherin* promoter in HBxAg-positive HepG2 cells (LIU et al., 2006). Accordingly, there may be a relationship between HBV infection and methylation status change in newborns. We hypothesized that HBV intrauterine infection could change DNA methylation status in newborns so as to have profound effect on their health. Therefore, we conducted this study to investigate whether different patterns of genome-wide DNA methylation can be detected in the umbilical cord blood of neonates who were exposed to maternal HBV infection during pregnancy, compared to healthy controls. The findings of this study may provide a new perspective for the practice of HBV prevention.

2. Materials & methods

2.1. Study population

Participants in this study were selected from a 1064-mother/child-dyads cohort collected between years of 2014 to 2015 at the Lianhua Hospital, in Xiamen City, China. Pregnant women were enrolled in the cohort if they met the criterions as follows: 1) living in the city at least 1 year; 2) natural pregnancy; 3) single birth; 4) gestational age ≤ 16 weeks at the time when the maternal health card was issued. Meanwhile, we excluded pregnant women with history of endocrine or metabolic diseases, liver or kidney disease, blood system diseases, genetic diseases, or occupational contact with toxic substances, and candidates whose husband was a HBV patient or HBV-carrier. Candidates receiving hormone therapy and using of assisted reproductive technologies, such as in vitro fertilization were also not enrolled into the study. After getting into the cohort, mothers were asked to complete a questionnaire and asked permission to abstract information from their pregnancy cards. The contents of questionnaire were mainly about demographic characteristics (age, height, pre-pregnancy BMI, education level, family annual income, and occupation), behaviors in early-stage of pregnancy (folate supplementary, smoking habits, alcohol consumption, and other pregnancy behaviors), history of pregnancy and pregnancy examination situation and etc. Then they were followed up until they gave birth to newborns. Meanwhile, information on the mode of delivery was also collected. Samples from peripheral venous blood of mothers were collected when they got into the study. After delivery, the umbilical cord blood was collected in an EDTA tube immediately and was used for analysis of HBV and genomic DNA methylation status.

Neonates were identified as intrauterine infection participants if HBsAg or HBV-DNA in cord blood was positive (Lanfang et al., 2014). After the HBsAg and HBV-DNA detection, we got 179 HBsAg-positive mothers of whom there were 34 newborns with intrauterine HBV infection. Excluding the candidates with pregnancy complications or adverse birth outcomes (e.g. gestational high blood pressure, gestational diabetes, depression and preterm birth), we selected 12 neonates with intrauterine infection whose mothers were HBsAg-positive. Twelve healthy neonate controls born to HBsAg-negative mothers, loosely matched on sex, maternal age and pre-pregnancy BMI, were also enrolled in the study.

2.2. Ethics

All participants signed the information consents before they got into the cohort. The study has been performed according to the World Medical Association Declaration of Helsinki and the procedures were approved by the ethics committee of the school of public health, Xiamen University.

2.3. Detailed definition of the participants' characteristics

Maternal age, pre-pregnancy BMI, educational level, family income, smoking habits, alcohol consumption, and folate supplementary were obtained from the questionnaire. Gender of neonates was abstracted from their birth cards. The missing message would be completed by asking the candidate again. Pre-pregnancy BMI (kg/m^2) was calculated as weight (kg) divided by the square of height (m^2). Educational level was classified into one of the four groups (< high school, high school, some college, and ≥ 4 years of college). Smoking habits were defined as smoking at least one cigarette every day for more than half of a year. Alcohol consumption was defined as having alcohol drinks (beers, wines or white spirits) at least once a week for more than half of a year. The time of folate supplementary was classified into never, before pregnancy, after pregnancy. Family annual income was divided into four levels (< 50 thousand, 50–100 thousand, 100–200 thousand, ≥ 200 thousand). Mode of delivery included caesarean delivery (CS) and unassisted vaginal delivery (VD).Only infants born at term, either by CS or VD, were eligible in the analysis.

2.4. HBsAg and HBV-DNA detection

The detection of HBsAg in the peripheral and cord blood was conducted by the method of enzyme linked immunosorbent assay (ELISA), using Hepatitis B virus surface antigen ELISA kit (Wantai Biological Pharmacy Enterprise, WB 2296, Beijing), while the load of HBV-DNA was measured using Hepatitis B Virus (HBV) PCR Kit (DAAN Gene, Guangzhou).

2.5. Sample collection, DNA extraction and quality check

All of the biological samples were stored at −80 °C. Genomic DNA was isolated from the cord blood using the QIAamp DNA Mini Kit (Qiagen, 51,306, German). The concentration and completeness of DNA were analyzed by the Qubit® 3.0 Fluorometer (Thermo Fisher Scientific, USA) and agarose gel electrophoresis, respectively.

2.6. Genome-wide DNA methylation assays

Bisulfite conversion of the DNA was performed in one bisulfite batch using the Zymo EZ DNA Methylation™ kit (Zymo, D5001, USA) following the manufacturer's protocol. After validating that unmethylated cytosine had converted to uracil using Universal Methylated Human DNA Standard (Zymo, D5011, USA) as controls, samples were sent to Shen Zhen Huada Gene Research Institute to amplify, fragment and hybridize the converted DNA to Beadchip. The twenty-four samples were randomized allocated to two hybridization chips (chip1 had 7 cases & 5 controls and chip2 had 5 cases & 7 controls). All of the converted DNA samples were processed on the same machine in the same day by the same technician to reduce batch effects. The Illumina Infinium Human Methylation 450K BeadChip array platform was