

Evaluation of neonatal Toll-like receptors 3 (c.1377C/T) and 9 (G2848A) gene polymorphisms in HBV intrauterine transmission susceptibility

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SUMMARY

To investigate whether single nucleotide polymorphisms (SNPs) in Toll-like receptors (TLRs) 3 and 9 affect the susceptibility of hepatitis B virus (HBV) intrauterine transmission, we genotyped 399 neonates for TLR3 (c.1377C/T) [rs3775290] and TLR9 (G2848A) [rs352140] using polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP). A femoral venous blood sample was obtained from these subjects. Hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) were measured using chemiluminescence immunoassay kits and hepatitis B virus DNA (HBV DNA) levels were determined by fluorescence quantitative PCR assay. Our results showed that when adjusting for maternal HBeAg, maternal HBV DNA and mode of delivery, allele ‘T’ for SNP c.1377C/T was significantly associated with HBV intrauterine transmission susceptibility [adjusted OR (aOR) 0.55, 95% confidence interval (CI) 0.34–0.91, $P = 0.020$] and the TT genotype decreased the risk of HBV intrauterine transmission (aOR 0.28, 95% CI 0.09–0.91, $P = 0.033$). Allele ‘A’ for SNP G2848A was significantly associated with HBV intrauterine transmission susceptibility (aOR 0.62, 95% CI 0.39–1.00, $P = 0.048$) and the GA genotype protected neonates from HBV intrauterine transmission (aOR 0.45, 95% CI 0.22–0.93, $P = 0.031$). The TLR3 (c.1377C/T) and TLR9 (G2848A) polymorphisms may be relevant for HBV intrauterine transmission susceptibility, although the reduction in risk to HBV intrauterine transmission is modest and the biological mechanism of the observed association merits further investigation.

Key words: HBV, intrauterine transmission, polymorphisms, Toll-like receptor.

INTRODUCTION

Hepatitis B virus (HBV) infection is a global public health problem and the most common cause of

acute and chronic liver disease worldwide [1, 2]. Mother-to-child transmissions, including intrauterine, labour, breastfeeding and daily contact, are major routes of HBV infection and account for about half of HBV carriers [3–5]. Furthermore, the rate of intrauterine transmission, which accounts for the majority of mother-to-child transmissions, in hepatitis B surface antigen (HBsAg)-positive pregnant women ranges from 5% to 40% in different areas of China

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[6, 7]. Hence, understanding the risk factors of HBV intrauterine transmission will help to effectively control the spread of HBV infection. Although intrauterine transmission remains to be elucidated, genetic susceptibility [8, 9] and various maternal characteristics (i.e. mode of delivery, history of abortion, antepartum haemorrhage, maternal HBV DNA load and serum markers of HBV infection during pregnancy) may all contribute to intrauterine transmission [10–12].

Toll-like receptors (TLRs), which act as bridging molecules between innate and adaptive immunity, are able to recognize different viruses and/or bacteria by pathogen-associated molecular patterns (PAMPs) and activate a pathogen-specific immune response [13, 14].

Single nucleotide polymorphisms (SNPs) are the most common form of genetic variants in the human genome, some of which have potential functional influence on susceptibility to human diseases. It is now becoming increasingly apparent that TLR gene polymorphisms have a considerable role in disease susceptibility [15–17]. Unlike other members of the TLR gene family that constitute the membrane-bound pattern recognition receptors, TLR3 and TLR9 are localized intracellularly, mostly in the endocytic compartments; TLR3 recognizes virus-derived dsRNA as well as poly I:C, a synthetic dsRNA analogue, while TLR9 recognizes unmethylated CpG motifs present in bacteria and viruses [18]. TLR3 mediates its functional effects via the myeloid differentiation primary response protein 88 (MyD88)-independent pathway using Toll-IL1R domain-containing adapter-inducing interferon- β (TRIF), resulting in anti-viral activity and/or apoptosis by induction of interferons and caspase recruitment. Alternatively, TLR9 functions through the MyD88-dependent pathway leading to NF- κ B activation, cytokine secretion and the inflammatory response [19]. Emerging evidence suggests a growing relevance of TLRs in the aetiopathogenesis of infectious disease [13–16]. The minor allele frequencies of TLR3 (c.1377C/T) and TLR9 (G2848A) in the Han Chinese in Taiwan were 38% and 44%, respectively [20]. Previous studies have reported these two SNPs were significantly associated with so many cancer susceptibilities such as cervical cancer, nasopharyngeal carcinoma as well as other cancers [19, 21]. The present study aimed to identify the role of TLR3 (c.1377C/T) [rs3775290] and TLR9 (G2848A) [rs352140] gene polymorphisms in HBV intrauterine transmission susceptibility.

MATERIALS AND METHODS

Study population

All of the 399 neonates recruited in the study, during June 2011 and July 2013, were from the Third People's Hospital of Taiyuan. All the neonates were born to HBsAg-positive mothers who were HCV and HIV negative, with normal liver function test (LFT). The mothers had not received any antiviral treatment during pregnancy.

Information was collected by trained interviewers using a standardized, structured questionnaire through face-to-face interviews. Maternal information included demographics and characteristics before and during pregnancy. Mode of delivery, maternal complications and newborns' information were obtained from medical records.

According to national recommendations, all neonates received one dose of 200 IU hepatitis B immune globulin (HBIG) and their first recombinant hepatitis B vaccine (NCPC GeneTech Biotechnology Co. Ltd, China) within 24 h after birth. Recombinant hepatitis B vaccination was completed with a further two doses of vaccine at ages 1 and 6 months.

Femoral venous blood samples of neonates was obtained before the administration of passive-active immunoprophylaxis. Peripheral blood samples from the mothers were obtained before delivery; all blood samples were collected using heparinized syringes. Non-anticoagulant peripheral blood samples from neonates (before the administration of passive-active immunoprophylaxis) and mothers (before delivery) were also collected.

The research protocol was approved by the Human Investigation Committee at the Shanxi Medical University (2010032). Informed written consent was obtained from all mothers.

Serological HBV markers and HBV DNA

HBsAg and hepatitis B e antigen (HBeAg) were measured using chemiluminescence immunoassay (CLIA) kits (Roche Co. Ltd, Switzerland) for all mothers during pregnancy, and their neonates on the day of delivery before the administration of passive-active immunoprophylaxis. HBV DNA levels of mothers were determined by fluorescence quantitative polymerase chain reaction (PCR) assay (Da'an Gene Co. Ltd, Sun Yat-Sen University, Guangdong, China). HBV DNA loads $> 1 \times 10^3$ copies/ml were defined as positive. HBV intrauterine transmission was defined

as finding HBsAg and/or HBV DNA positive in the peripheral blood of neonates within 24 h after birth, before active or passive immunoprophylaxis [22]. Newborns with HBV intrauterine transmission were considered cases, those without were considered controls.

DNA extraction

Genomic DNA extraction from peripheral blood samples was performed using RelaxGene Blood DNA System (Tiangen Biotechnologies Co. Ltd, China).

Genotyping for TLR3 (c.1377C/T) [rs3775290] and TLR9 (G2848A) [rs352140] gene polymorphisms

Polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) was performed to identify TLR3 (c.1377C/T) and TLR9 (G2848A) gene polymorphisms in intrauterine transmission cases and control subjects. The validation of PCR–RFLP has been conducted in many studies [9, 20, 23–25], and the comparative studies on the assay method for genotyping using both PCR–RFLP and DNA sequence analysis demonstrated that there was considerable concordance between the results of both assay techniques [26, 27]. PCR for TLR3 (c.1377C/T) was performed in a total reaction volume of 25 µl with 20 pmol each of forward and reverse primers (F) 5'-CCAGGCATA AAAAGCAATATG and (R) 5'-GGACCAAGGC AAAGGAGTTC [19], genomic DNA (200 ng) and 2 × PCR Master Mix (MBI Fermentas, USA). PCR conditions were as follows: initial denaturation of 95 °C for 5 min, 35 cycles of 95 °C for 45 s, 55 °C for 45 s, and 72 °C for 30 s, followed by a final extension for 7 min at 72 °C. All these reactions were performed in a thermal cycler (Eppendorf, Germany). The PCR products were digested by restriction endonuclease *TaqI* (MBI Fermentas) at 65 °C overnight and then analysed by 10% polyacrylamide gel electrophoresis. Bands of 274 and 63 bp corresponded to TLR3 CC while 337, 274 and 63 bp bands were designated as heterozygous CT individuals; a band of 337 bp corresponded to the homozygous TT genotype.

PCR for TLR9 (G2848A) was performed in a total reaction volume of 25 µl with 20 pmol each of forward and reverse primers (F) 5'-AAGCTGGA-CCTCTACCACGA and (R) 5'-TTGGCTGTG-GATGTTGTT [19], genomic DNA (200 ng) and 2 × PCR Master Mix (MBI Fermentas). PCR conditions were as follows: initial denaturation of 95 °C for 5 min, 35 cycles of 95 °C for 45 s, 56 °C for 1 min, and 72 °C

for 30 s, followed by a final extension for 7 min at 72 °C. All these reactions were performed in thermal cycler (Eppendorf). The PCR products were digested by restriction endonuclease *BstUI* (MBI Fermentas) at 60 °C overnight and then analysed by 10% polyacrylamide gel electrophoresis. The undigested PCR product was of 327 bp; upon *BstUI* digestion, bands of 190 and 137 bp corresponded to TLR9 GG genotype while bands of 327, 190 and 137 bp were designated as heterozygous GA; a band of 327 bp corresponded to the homozygous AA genotype.

Statistical analysis

By univariate analyses, χ^2 tests were used for categorical data and Student's *t* tests were used for continuous variables. Allele frequencies and genotype distributions for each SNP were descriptively summarized using frequencies and percentages. The Hardy–Weinberg equilibrium for TLR genotypes was performed by χ^2 test. The association between genotyped polymorphisms and the risk of disease was estimated by odds ratio (OR), 95% confidence interval (CI) and *P* values. A *P* value of <0.05 was considered to indicate statistical significance. A multivariable logistic regression analysis was performed to adjust risk factors (such as maternal HBeAg, maternal HBV DNA and caesarean section) using SPSS v. 16.0 (SPSS Inc., USA). The logistic regression model also was used to analyse the interaction between neonatal TLR3 (c.1377C/T) and TLR9 (G2848A) gene polymorphisms and maternal HBeAg, maternal HBV DNA and mode of delivery.

RESULTS

Demographic and clinical characteristics of study subjects

The study detected 51 newborns with serum HBsAg or HBV DNA positive, the incidence of HBV intrauterine transmission was 12.78% (51/399). The demographic and clinical characteristics of study subjects are summarized in Table 1. As shown in Table 1, distributions of maternal and newborn characteristics were similar across cases and controls.

Associations between maternal characteristics and HBV intrauterine transmission

By univariate analyses (Table 2), maternal HBeAg positivity (*P* = 0.001), maternal HBV DNA positivity

Table 1. Distributions of selected maternal and newborn characteristics in cases and controls

Characteristics	Cases (N = 51) n (%)	Controls (N = 348) n (%)	P value
Maternal characteristics			
Age (years)			
Mean (\pm S.D.)	26.82 (4.82)	27.69 (4.34)	0.190
Newborn characteristics			
Birth weight (g)			
2500–3999	48 (94.1)	309 (88.8)	0.247
\geq 4000	3 (5.9)	39 (11.2)	
Gestational weeks			
<37	1 (2.0)	10 (2.9)	0.888
37–41	48 (94.1)	321 (92.2)	
>41	2 (3.9)	17 (4.9)	
Gender			
Girl	25 (49.0)	189 (54.3)	0.479
Boy	26 (51.0)	159 (45.7)	

($P = 0.001$) and caesarean section ($P < 0.001$) were significantly associated with HBV intrauterine transmission.

Associations between TLR3 (c.1377C/T) and TLR9 (G2848A) gene polymorphisms and HBV intrauterine transmission

The observed genotype and allele frequency distribution of TLR3 (c.1377C/T) and TLR9 (G2848A) gene polymorphisms between intrauterine transmission cases and controls are shown in Table 3. The genotype frequencies were in Hardy–Weinberg equilibrium. After adjusting for important covariates (i.e. maternal HBeAg, maternal HBV DNA and mode of delivery), minor allele ‘T’ of TLR3 was significantly associated with HBV intrauterine transmission susceptibility [adjusted odds ratio (aOR) 0.55, 95% confidence interval (CI) 0.34–0.91, $P = 0.020$] and the TT genotype decreased the risk of HBV intrauterine transmission (aOR 0.28, 95% CI 0.09–0.91, $P = 0.033$). Carrier analysis demonstrated that TLR3 CT + TT was significantly associated (aOR 0.52, 95% CI 0.27–0.99, $P = 0.046$) with intrauterine transmission susceptibility. Minor allele ‘A’ of TLR9 was significantly associated with HBV intrauterine transmission susceptibility (aOR 0.62, 95% CI 0.39–1.00, $P = 0.048$) and the GA genotype protected neonates from HBV intrauterine transmission (aOR 0.45, 95% CI 0.22–0.93, $P = 0.031$). Carrier analysis

also revealed that TLR9 GA+AA was significantly related (aOR 0.44, 95% CI 0.22–0.88, $P = 0.019$) to intrauterine transmission susceptibility.

We also performed interaction analysis of statistics, the results suggested there was an interaction between caesarean section and TLR3 CC and TT on intrauterine transmission susceptibility; and there was interaction between maternal HBeAg positivity and TLR9 GG, GA and AA on intrauterine transmission susceptibility.

DISCUSSION

As we know, neonates born to HBsAg-positive mothers, as a special population, are in danger of immunity failure by immunoprophylaxis and chronic infection by HBV. Therefore, the situation attracted greater attention from physicians and public health experts whether it was HBV intrauterine transmission or not [28].

In China, one of the major reasons for the high prevalence rate of HBV infection is that mothers transmit HBV to their children during the intrauterine as well as the perinatal periods. About 50% of HBV carriers became infected via mother-to-infant transmission. With the immunization of hepatitis B vaccine and the use of HBIG in HBsAg-positive pregnant women, the incidence rate of HBV carrier status in children has fallen markedly. However, HBV intrauterine transmission still occurs at a high incidence rate. The exact mechanisms underlying HBV intrauterine transmission have not been completely elucidated [8].

Apart from several investigated factors (i.e. mode of delivery, history of abortion, antepartum haemorrhage, maternal HBeAg positivity, maternal HBV DNA load) that have been reported to be related to HBV intrauterine transmission [3], recent studies have indicated that genetic background also influences the susceptibility to HBV intrauterine transmission [8, 9].

TLRs play pivotal roles in the immune system and initiate inflammatory response to foreign pathogens including viruses, bacteria and fungi [29], and they are emerging as plausible susceptibility markers in several infectious diseases or immune disorders. Gene variants of the potential antiviral TLRs (TLR3 and TLR9) certainly have relevance in terms of susceptibility to epidemical viral infection diseases [20]. In this study, we assessed whether polymorphisms in TLR3 (c.1377C/T) and TLR9 (G2848A) are associated with individual susceptibility to HBV intrauterine transmission.

Table 2. Univariate analyses of associations between maternal characteristics and HBV intrauterine transmission

Characteristics	Cases		Controls		P value
	No n (%)	Yes n (%)	No n (%)	Yes n (%)	
Pre-pregnancy					
HBV vaccine injection	46 (90.2)	5 (9.8)	300 (86.2)	48 (13.8)	0.433
Menstrual regularity	1 (2.0)	50 (98.0)	19 (5.5)	329 (94.5)	0.468
History of abortion	29 (56.9)	22 (43.1)	202 (58.0)	146 (42.0)	0.873
Family history of HBV infection	37 (72.5)	14 (27.5)	238 (68.4)	110 (31.6)	0.549
Pregnancy					
Maternal HBeAg positive	14 (27.5)	37 (72.5)	193 (55.5)	155 (44.5)	0.001
Maternal HBV DNA positive	14 (27.5)	37 (72.5)	187 (53.7)	161 (46.3)	0.001
HBIG injection	45 (88.2)	6 (11.8)	301 (86.5)	47 (13.5)	0.732
HBV vaccine injection	50 (98.0)	1 (2.0)	345 (99.1)	3 (0.9)	0.462
Medication use during pregnancy	37 (72.5)	14 (27.5)	272 (78.2)	76 (21.8)	0.371
Pregnancy reaction	36 (70.6)	15 (29.4)	230 (66.1)	118 (33.9)	0.525
Pregnancy-induced hypertension	49 (96.1)	2 (3.9)	339 (97.4)	9 (2.6)	0.586
Antepartum haemorrhage	50 (98.0)	1 (2.0)	327 (94.0)	21 (6.0)	0.234
Delivery					
Caesarean section	40 (78.4)	11 (21.6)	142 (40.8)	206 (59.2)	<0.001
Amniotic fluid pollution	47 (92.2)	4 (7.8)	302 (86.8)	46 (13.2)	0.279
Abnormality of umbilical cord	17 (33.3)	34 (66.7)	124 (35.6)	224 (64.4)	0.748
Praevia placenta	51 (100.0)	0 (0.0)	346 (99.4)	2 (0.6)	1.000

HBV, Hepatitis B virus; HBeAg, hepatitis B e antigen; HBIG, hepatitis B immunoglobulin; HBV DNA, hepatitis B virus DNA.

Table 3. Genotype and allele frequency distribution of neonatal TLR3 (*c.1377C/T*) [*rs3775290*] and TLR9 (*G2848A*) [*rs352140*] gene polymorphisms in HBV intrauterine transmission

TLR gene polymorphisms	Cases (%) (n = 51)	Controls (%) (n = 348)	P value	OR (95% CI)
TLR3				
CC	21 (41.2)	95 (27.3)	—	Reference
CT	26 (51)	190 (54.6)	0.125	0.59 (0.30–1.16)
TT	4 (7.8)	63 (18.1)	0.033	0.28 (0.09–0.91)
CT+TT	30 (58.8)	253 (72.7)	0.046	0.52 (0.27–0.99)
C*	68 (66.7)	380 (54.6)	—	Reference
T*	34 (33.3)	316 (45.4)	0.020	0.55 (0.34–0.91)
TLR9				
GG	18 (35.3)	82 (23.6)	—	Reference
GA	23 (45.1)	192 (55.2)	0.031	0.45 (0.22–0.93)
AA	10 (19.6)	74 (21.3)	0.063	0.42 (0.17–1.05)
GA+AA	33 (64.7)	266 (76.4)	0.019	0.44 (0.22–0.88)
G*	59 (57.8)	356 (51.1)	—	Reference
A*	43 (42.2)	340 (48.9)	0.048	0.62 (0.39–1.00)

OR, Odds ratio; CI, confidence interval.

ORs were adjusted by maternal HBeAg, maternal HBV DNA and mode of delivery by multivariable logistic regression analysis with SPSS v. 16.0 (SPSS Inc., USA).

* For alleles, total number of chromosomes in cases = 102 and in controls = 696.

The non-synonymous 1377C/T polymorphism present in exon 4 of the TLR3 gene on chromosome 4 affects the receptor–ligand interaction by altering the

TLR3 ectodomain and functionally impairing the receptor. The minor allele frequency of TLR3 (*c.1377C/T*) in our control population was 45.4%.

By contrast, in the Han Chinese in Taiwan and Japanese populations it was 38% and 40%, respectively [20].

As HBV intrauterine transmission is a multifactorial disease, we also assessed the interaction of this SNP when adjusting for maternal HBeAg, maternal HBV DNA and mode of delivery. We observed an increased risk of intrauterine transmission associated with HBeAg-positive women and those who were HBV DNA positive, which was consistent with the majority of early studies [3, 30–32]. We also discovered that caesarean delivery was protective for HBV intrauterine transmission, which is consistent with much earlier studies [3, 32]. Multivariable logistic regression analysis was performed to adjust these risk factors such as maternal HBeAg, maternal HBV DNA and mode of delivery. Minor allele 'T' of TLR3 was significantly associated with HBV intrauterine transmission susceptibility (aOR 0.55, 95% CI 0.34–0.91, $P = 0.020$) and the TT genotype decreased the risk of HBV intrauterine transmission (aOR 0.28, 95% CI 0.09–0.91, $P = 0.033$). Carrier analysis demonstrated that TLR3 CT+TT was significantly associated (aOR 0.52, 95% CI 0.27–0.99, $P = 0.046$) with intrauterine transmission susceptibility. The precise biological mechanism of the observed association needs further investigation.

The synonymous +2848 G > A polymorphism is present in exon 2 of the TLR9 gene and possibly affects expression at the mRNA level. TLR9 functions through the MyD88-dependent pathway leading to NF- κ B activation, cytokine secretion and the inflammatory response [19]. Viruses with a DNA genome, such as herpesviruses, may potentially activate TLR9, which is activated in response to CpG DNA, to induce cytokine expression [20]. Minor allele frequency of 48.9% was observed in the control study population. Previous studies evaluating the association of TLR9 (G2848A) gene polymorphisms with disease susceptibility in Japanese, Chinese, German and European American populations have reported minor allele frequencies of 34%, 44%, 55.4% and 57%, respectively [20, 33–35]. These research findings all suggest the relevance of host genetic factors in inter-individual differences to disease susceptibility. After adjusting risk factors such as maternal HBeAg, maternal HBV DNA and mode of delivery, the minor allele 'A' of TLR9 was significantly associated with HBV intrauterine transmission susceptibility (aOR 0.62, 95% CI 0.39–1.00, $P = 0.048$), and the GA genotype protected neonates from HBV

intrauterine transmission (aOR 0.45, 95% CI 0.22–0.93, $P = 0.031$). The AA genotype of TLR9 showed borderline association with HBV intrauterine transmission (aOR 0.42, 95% CI 0.17–1.05, $P = 0.063$). Carrier analysis demonstrated that TLR9 GA+AA was significantly associated (aOR 0.44, 95% CI 0.22–0.88, $P = 0.019$) with intrauterine transmission susceptibility. Additional studies with a larger sample size may be required to understand the role of TLR9 gene polymorphism in HBV intrauterine transmission in neonates.

So far, there is a paucity of data regarding the associational and functional implications of common TLR3 and TLR9 gene variants in human cancers: TLR3 gene polymorphisms have also been implicated in nasopharyngeal carcinoma risk in the Cantonese population [30]. The role of TLR9 (G2848A) gene polymorphism in the pathogenesis of Hodgkin's lymphoma has been previously investigated and the findings demonstrated that polymorphisms and haplotypes in TLR9 and MyD88 are associated with the development of Hodgkin's lymphoma [36]. However, very few papers on the relationship between TLR3 and TLR9 gene polymorphisms and susceptibility to HBV intrauterine infection have been reported. To the best of our knowledge, this is the first report involving a study on the association between TLR3 (c.1377C/T) and TLR9 (G2848A) gene polymorphisms and the outcomes of HBV intrauterine transmission in newborn infants. Our findings support the conclusion that genetic factors are associated with the outcomes of HBV intrauterine transmission. The characterization of functional significance of this particular TLR molecule in HBV intrauterine transmission may provide insight leading to the development of novel prophylactic and therapeutic approaches. Further study is still needed to validate these findings and to further explore the relationship between HBV intrauterine transmission and the susceptible gene(s), as well as any other risk factors which may be involved in HBV intrauterine transmission. Finally, investigation to elucidate the genetic pathogenesis of HBV intrauterine transmission is among the future studies we plan to pursue.

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DECLARATION OF INTEREST

None.

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