Relationship of Placental TLR-7 Expression with Cord Blood HBV DNA and Placental HBV DNA

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Abstract

Objective: To determine the role of TLR-7 expression on intrauterine vertical transmission in pregnancy through identification of serum hepatitis B markers in both maternal and umbilical cord blood.

Method: Analysis of TLR expression was performed on 38 paraffin block samples of placental tissue acquired from mothers with HBV using TLR immunohistochemical staining.

Result: 16 of 38 samples were acquired from mothers aged 26-30 years old. Most of the samples were from primiparous mothers (52.6%). This study found no significant association between TLR-7 expression and HBV DNA in the placenta and cord blood (p=1.000). However, we found a significant association between placental TLR-7 expression and maternal HBV DNA (p=0.034). Meanwhile, placental HBeAg and HBV DNA were not associated with placental TLR-7 expression (p=0.082; p = 1.000).

Conclusion: There was no significant association between TLR-7 expression and HBV DNA in the placenta and cord blood, but we found a significant association between TLR-7 expression and maternal HBV DNA.

Key word: toll-like receptor (TLR) 7, HBV DNA, umbilical cord, placental, Hepatitis B, intrauterine infection

Hubungan Ekspresi TLR-7 Plasenta dengan HBV DNA Tali Pusat dan HBV DNA Plasenta

Abstrak

Tujuan: Melihat peran ekspresi TLR-7 terhadap transmisi vertikal intrauterina pada kehamilan melalui identifikasi marker serum hepatitis B pada darah ibu dan talipusar.

Metode: Analisis ekspresi TLR dilakukan pada 38 sampel blok paraffin jaringan plasenta ibu yang menderita HBV dengan memakai pewarnaan imuhohistokimia TLR.

Hasil: 16 dari 38 sampel berusia 26-30 tahun. Sebagian besar sampel merupakan kelompok primipara (52.6%). Penelitian ini tidak menemukan hubungan yang signifikan antara ekspresi TLR-7 di plasenta dan HBV DNA darah tali pusat (p=1.000). Tapi, kami menemukan hubungan yang signifikan antara ekspresi TLR-7 plasenta dan HBV DNA ibu (p=0,034). Sedangkan HBeAg dan HBV DNA plasenta tidak berhubungan dengan ekspresi TLR-7 plasenta (p=0,082; p=1.000).

Kesimpulan: Tidak ada hubungan yang signifikan antara ekspresi TLR-7 dan DNA HBV di plasenta dan tali pusat, tetapi kami menemukan hubungan yang signifikan antara ekspresi TLR-7 dan DNA HBV ibu.

Kata kunci: Toll-like receptor (TLR) 7, HBV DNA, tali pusat, plasenta, Hepatitis B, infeksi intrauterina

Introduction

Hepatitis B virus (HBV) infection is a serious public health problem, where more than one million people die every year due to this disease. Chronic HBV infection affects more than 350 million people worldwide, leading to a range of liver diseases, including chronic hepatitis, fulminant liver failure, cirrhosis of the liver, and hepatocellular carcinoma.[1]

Chronic hepatitis B, as a worldwide health problem, is a disease that begins in the prenatal period, and its complications gradually become apparent later in life.[2,3] The prevalence of HBV in pregnant women is about 5% and varies from 0.6% in low-incidence areas to more than 20% in areas with a higher incidence such as eastern and African regions.[2] Indonesia is considered as a country with a high incidence of hepatitis along with 11 other countries in Southeast Asia. The Indonesian Health Profile data for 2018 further shows that the national prevalence of Hepatitis B in pregnant women reaches 1.88%. South Sulawesi Province is ranked 12th among other Indonesian provinces for having the most pregnant women with hepatitis B (2.51%).[4]

Cheung et al. divide the hepatitis B vertical transmission mechanism into three gestational periods: 1) at conception where germ-line infection occurs; 2) during pregnancy through contamination of maternal blood and transplacental transmission, and 3) at birth via membrane rupture and vaginal delivery. The transmission rate through these three mechanisms is associated with positive HBeAg status and high levels of Hepatitis B Virus (HBV) DNA.[5,6]

There are many serum markers to indicate the risk of vertical transmission of hepatitis B virus via intrauterine such as maternal viral load and HBeAg. High viral load values indicate an increased risk of vertical transmission and positive HBeAg. Both of these are closely related to the levels of HBV DNA in cord blood.[7] Based on research conducted by Nie in 2011, intrauterine infection and vertical transmission occur when HBV DNA titer is detected in neonatal peripheral venous blood or umbilical cord blood.[8] However, studies suggest that the detection of HBV DNA levels in umbilical cord blood is not considered as an absolute marker of intrauterine infection but suggests a risk of hepatitis B virus transmission in the placenta.[9]

Infants whose mothers were HBeAg positive and cord blood HBV DNA positive but at the time of placental examination did not develop an intrauterine infection suggests that there is an unexplained mechanism that appears to protect the fetus from intrauterine HBV infection. The mechanism by which HBV passes across the placental barrier and infects the fetus is still unknown. However, it has been demonstrated that HBV infects trophoblasts in vivo and in vitro, and this infection is the first and most crucial step of intrauterine HBV infection.[1]

The placenta is considered a specific component during the pregnancy which has an innate immune system and contains mechanical and immunological barriers restricting entry to the fetus. A study revealed the function of placental trophoblasts as macrophages in recognizing and responding to pathogens through the expression of toll-like receptors (TLRs), and primary placental trophoblasts are more resistant to viral infection than non-trophoblastic cells. The mechanism by which placental trophoblasts combat viruses, including HBV, needs to be elucidated. However, TLRs on trophoblasts can play a vital role.[1] Based on this background, research is needed to evaluate the role of TLR-7 expression on intrauterine vertical transmission in pregnancy through the identification of serum hepatitis B markers in maternal and umbilical cord blood so that in the future, the necessary treatment can be given to reduce mother-

to-child HBV transmission. Research data regarding placental TLR-7 expression on umbilical cord hepatitis B virus detection in Indonesia, especially in Makassar, has never been reported to the best of the authors' knowledge.

Method

This study is a cross-sectional diagnostic study and is conducted in four hospitals in Makassar from September 2019 to March 2020. Our population consisted of HbsAg-positive pregnant women in Makassar. In this study, the inclusion criteria were women with term pregnancy who were infected with hepatitis B virus with positive HBsAg test results, who had never received antiviral therapy before, were willing to participate in the study by providing written consent after receiving thorough explanation of the study. The exclusion criteria in this study were women who received antiviral therapy within six months to recruitment; were receiving interferon therapy; had pregnancy complications (preterm labor, premature rupture of membranes, and preeclampsia); fetal distress; had an autoimmune disease, HIV, syphilis, or other infectious diseases; and had abnormal liver and kidney function. Incomplete data including those who did not follow all required procedures as well as those who withdrew form this study for any reason were not included in our analysis. Sampling was carried out using the purposive sampling technique. The number of samples in this study was 38 subjects. Data analysis used STATA 14. Univariate analysis was conducted to determine baseline characteristics, while the relationship between placental TLR-7 expressions and maternal, umbilical cord, and placental HBV-DNA were analyzed using the Fischer-exact test.

Instruments and Procedures

All pregnant women who met the inclusion criteria described above who have agreed to participate in this study were asked to sign an informed consent form. We conducted history taking, physical examination, and laboratory tests (HBsAg and HBV DNA) on all participants. Pregnant women who have previously been diagnosed with HBV infection confirmed by a positive HBsAg result during pregnancy, then had their blood samples took and analyzed for HBV markers. Blood samples were taken when the patient was admitted to the hospital. The blood sample is then centrifuged after leaving it for at least 30 minutes at room temperature. HBsAg markers were routinely examined using ELISA (enzyme-linked immunosorbent assays), and HBV DNA levels were assessed using real-time quantitative PCR.

After the baby is born, either by vaginal delivery or surgery, the umbilical cord blood is collected after cutting the cord and before the placenta is separated from the uterus for HBV DNA examination. HBV DNA markers were quantified using a qualitative serological test. A placenta sample is taken as soon as the placenta is delivered. The placental cotyledons were dissected in the middle zone, washed thoroughly with cold normal saline after separation from the amniotic membrane, decidua, and connective tissue. Then frozen with liquid nitrogen and then stored at -800C until ready for inspection. The tissue in the paraffin block was cut to a size of 5 μm and glued to a poly-L-lysine slide, and then deparaffinized. Immunohistochemical staining was carried out using the standard avidin-biotin-peroxidase complex (ABC) method. The unstained slides were incubated with peroxidase-1 for 5 minutes at room temperature, after which an ABC procedure was followed. Immunohistochemical staining used monoclonal TLR-7 antibody concentrated with a 1:100 dilution. The

results of immunohistochemical staining were evaluated using light microscopy by two pathologists and researchers.

Result

This study involved 38 pregnant women who had hepatitis B based on history taking, physical examination, and confirmed by laboratory examination. Sample characteristics were grouped according to age, parity, gestational age, and hepatitis risk factors, including a history of hepatitis and family history of hepatitis.

We divided our samples into five age groups: <20 years-old, 21-25 years-old, 26-30 years-old, 31-35 years-old, and> 35 years-old. Most of our samples were in the 26-30 years-old age group (42.1%), and only one sample was in the <20 years-old age group (2.6%). In this study, the youngest patient was 18 years-old, and the oldest was 39 years-old with a mean ± standard deviation of 28.78 ± 4.70 years. More than half of our samples were primiparous (52.6%), and two samples were grand-multiparous. About 34.2% of our samples had a history of hepatitis, and only two subjects had a family history of hepatitis (Table 1).

Based on the following table, it can be seen that there is no significant relationship between HBV-DNA cord blood levels and TLR-7 expression in the placenta with a p-value = 1.000 (p> 0.05) (Table 2).

Table 1 Sample Characteristics

Variables	Frequency (n=38)	Percentage (%)
Age		
<20 years-old	1	2.6
21-25 years-old	8	21.1
26-30 years-old	16	42.1
31-35 years-old	9	23.7
>35 years-old	4	10.5
Parity		
Primiparous	20	52.6
Multiparous	16	42.1
Grand-multiparous	2	5.3
History of Hepatitis		
Yes	13	34.2
No	25	65.8
Family History of Hepatitis		
Yes	2	5.3
No	36	94.7

Based on table 3, it can be seen that there is a significant relationship between the risk of transmission based on maternal HBV-DNA levels and the expression of TLR-7 in the placenta with a p-value value of 0.034 (p <0.05). There was no significant relationship between HBeAg levels and TLR-7 expression in the placenta where the p-value was 0.118 (p> 0.05). There was no significant relationship between placental HBV-DNA levels and TLR-7 expression in the placenta where the p-value was 1.000 (p> 0.05).

Table 2 Relationship between HBV-DNA Cord Blood Levels and TLR-7 Expression

Variable		HBV-DNA Cord Blood		Total	*p
Positive			Negative		
Positive TLR-7 expression	n	2	23	25	
	%	8%	92%	100.0%	
Negative TLR-7 expression	n	1	12	13	1.000
	%	7.7%	92.3%	100.0%	
Total	n	3	25	38	

*Fisher-exact test

Table 3 Relationship between Risk of Intrauterine HBV Exposure Based on Maternal HBV-DNA, HbeAg and Placental HBV-DNA Levels with TLR-7 Expression

Variable		TLR-7 expression		Total	P-value*
		Positive	Negative		
Maternal HBV-DNA	Positive	8 (100.0%)	0 (0.0%)	8 (21.1%)	**0.034**
	Negative	17 (56.7%)	13 (43.3%)	30 (78.9%)	
HBeAg	Positive	9 (90.0%)	1 (10.0%)	10 (26.3%)	0,082
	Negative	16 (57.1%)	12 (42.9%)	28 (73.7%)	
Placental HBV-DNA	Positive	9 (69.2%)	4 (30.8%)	13 (34.2%)	1.000
	Negative	16 (64.0%)	9 (36.0%)	25 (65.8%)	

*Fisher-exact test

Discussion

Sample Characteristics

Our samples were mostly obtained from mothers aged between 26-30 years-old (16 people (42.1%)), and the least were aged <20 years-old (one person (2.6%)). This finding is in line with a research conducted by Kolawole et al. in Nigeria, where the highest incidence of HBsAg was found among those within the 30 to 34-year-old age group with a percentage of 23.3%—followed by the second largest age group, the 25 to 29-year-old age group with a percentage of 16.9%. According to Kolawole et al., this age group has the highest peak of social activity or is considered the productive age, therefore that the risk of virus transmission through sexual contact is also fairly high.[10]

Based on parity status, a majority of our samples consisted of primiparous women (20 people (52.6%)), and in the grand-multiparous group, only two samples (5.3%) were obtained. Whereas in a study conducted by Dortey et al. reported that the prevalence of HBV-positive was more common in multiparous women due to repeated pregnancy and labor, which put pregnant women at a greater risk of HBV infection due to examination procedures and childbirth.[11] However, in a study conducted by Luuse et al., it was found that there was no difference between primiparous and multiparous HbsAg-positive women.[12]

The relationship between TLR-7 expression and the risk of vertical transmission by examining cord blood HBV DNA

Based on table 2, there was no significant relationship between cord blood HBV-DNA levels and TLR-7 expression in the placenta. However, we found that the percentage of mothers with a negative cord blood HBV-DNA gave more positive TLR-7 expression results. Our finding is in line with Tian et al.'s study where mothers infected with the hepatitis B virus who gave birth to infants without a HBV infection (infants who were not intrauterine infected) had higher levels

of TLR-7 expression than infants with an intrauterine infection.[1]

The factor underlying the insignificant relationship in this study was the maturity of the endosomal to express TLR-7. Endosomal immaturity is influenced by several genes and maternal immunity factors that may differ in our samples. TLR-7 is expressed in the intracellular compartment such as the endosome, by hence to identify ligands, and ligands require endosomal maturation recognized by hepatitis B. Thus the expression of TLR 3,7,8, and 9 can be used as parameters of infection or exposure to hepatitis B virus.[13]

We found that based on the percentage of positive TLR-7 expression, a majority had a negative cord blood HBV-DNA. This occurs where positive HBV DNA indicates HBV exposure to the placenta (risk of transmission), not an intrauterine infection. Because it has crossed the placental barrier generally increases TLR-7 expression. TLR-7, in particular, has an intracellular signaling mechanism by recognizing nucleic acids from the virus when it infects cells. Viruses that penetrate cells activate IFN. IFN activation will result in the expression of TLR-7 on lysosomes which will signal inflammatory cytokines and chemokines, which will inhibit transcription of viral RNA. The inhibition of viral transcription resulted in a decline of viral load.[9,14,15]altered TLR7 expression levels are implicated in various autoimmune disorders, indicating a key role for this receptor in modulating inflammation. This review is focused on the regulation of TLR7 expression and localization compared to that of the other endosomal TLRs: TLR3, 8, and 9. Endosomal TLR localization is a tightly controlled and intricate process with some shared components among various TLRs. However, TLR-specific mechanisms must also be in place in order to regulate the induction of pathogen- and cell-specific responses. It is known that TLR7 is shuttled from the endoplasmic reticulum to the endosome via vesicles from the Golgi. Several chaperone proteins are required for this process, most notably uncoordinated 93 homolog B1 (Caenorhabditis elegans

The Relationship between TLR-7 expression and the Risk of Vertical Transmission Through Examination of Maternal HBV DNA, HBeAg, and Placental HBV DNA

In this study, it was found that there was a significant correlation between HBV DNA titers >200,000 IU/ml and the expression of TLR-7 levels (Table 3). This finding is in line with a study conducted by Tian et al. where the expression of placental TLR-7 and TLR-8 was significantly increased in women with positive HBV DNA. Significant results in this study occurred as a result of the immune response to HBV by the placental trophoblast, which is the center for preventing intrauterine infection of HBV through upregulation of TLR7 as an immune response to HBV infection.[1] HBV DNA >2x 10^5 has a high risk of intrauterine infection affecting the placental immune response through a positive expression of TLR-7 on the placenta. Isogawa et al. conducted an in vivo study in mice and found a significant effect of TLR-7 expression on HBV virus replication. TLR-3, TLR-4, TLR-5, TLR-7, and TLR-9 all modulate replication against hepatitis B virus.[16]

A similar study conducted by Gao et al. revealed a significant relationship between TLR-9 and TLR-3 expression and the risk of intrauterine infection. Gao et al. described the influence of the role of genes in the risk of vertical transmission and their relationship with TLR-3 and TLR-9 expression. That large sample size was required to determine the mechanism of TLR expression and its relationship to intrauterine transmission.[17]

We also found no significant relationship between maternal HBeAg levels and TLR-7 expression in the placenta, as shown in

Table 3. This is likely due to genetic and immunity factors where the number of APC cells, such as dendritic cells is produced less or still immature. The Tfh cell and B-cell related gene factors affect the number of APC cells, which will later affect the expression of TLR-7 because it is known that TLR-7 is found in the intracellular antigen-presenting cell (APC), especially in the endosomal. In immature APCs, there is also an irregularity in the activation signal for TLR-7.[18]

Another similar study was also conducted by Wu et al. In that study, and it also assessed the relationship between placental Toll-Like receptor-3 with HbeAg among HbeAg-positive mothers. In this study, a negative correlation was found between a positive-HBeAg status and TLR-3, where the expression of TLR-3 decreased in mothers with positive HBeAg. However, this study did not explain the mechanism underlying the decrease in placental TLR-3 expression.[19] Research on maternal HBeAg and its relation to TLR-7 expression is still lacking, therefore further research is needed in this regard.

In this study, there was no significant relationship between placental HBV-DNA levels and the expression of TLR-7 in the placenta (see table 3). Our findings contradicted other studies including one by Tian, where it was found that there was an increase in placental TLR-7 expression levels in placental trophoblast cells exposed to hepatitis B virus in vitro.[1]

The insignificant association between placental HBV DNA levels and TLR-7 activation occurred because TLRs belong to the pattern recognition receptor (PRR) family, responsible for pathogen-related molecular inheritance pattern recognition (PAMPs). PAMP is a protein, lipid, polysaccharide, DNA, or RNA present in the membrane or envelope of pathogenic microorganisms. TLR is also able to recognize damage-related molecular patterns (DAMPs).[20] Based on the ligand domain that TLR can recognize, TLR-7 and TLR-8 can recognize ssRNA, and TLR-9 can recognize viral DNA ligands, while in our study the identified virus was hepatitis B virus, which is a dsDNA virus. Therefore, based on the mechanism of ligand recognition, it appears that TLR-7 is not expressed at the time of exposure to hepatitis B virus−a dsDNA virus that TLR-9 generally recognizes in the placenta.[13]

The limitation of this study is that we did not conduct an analysis related to maternal genetic factors, considering that genetic factors play a role in the immune response mechanism and its relation to TLR-7 expression. This study also did not take HBV DNA or HBsAg samples directly from infants to determine the risk of vertical transmission and did not follow-up on infants whose mothers had a high risk of HBV exposure. Further research is needed to explain the association of HBV DNA levels in the mother, placenta, and cord blood with the expression of TLR-7 as assessed quantitatively. Proof of intrauterine infection by examining samples in infants such as HBsAg and HBV viral load is required in future studies. Other factors related to the risk of vertical transmissions such as genetic factors, history of anti-hepatitis B treatment, clinical symptoms, and liver function can affect the expression of TLR-7 and should be further studied and investigated.

Conclusion

We did not find a significant association between TLR-7 and HBV DNA expression in either the placenta or cord blood. However, we did find a significant association between the expression of TLR-7 and maternal HBV DNA.

Conflict of Interest

The authors declare that they have no conflict

of interest concerning this study.

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