CD14/-159 and TNFα/-308 Promoter Polymorphisms are not associated with Development of Idiopathic Neonatal Hepatitis among Filipinos

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ABSTRACT

Objective. To determine if the CD14/-159 and the TNFα/-308 single nucleotide polymorphisms (SNPs) are associated with the development of Idiopathic Neonatal Hepatitis (INH) in Filipino children.

Methods. Genomic DNA from 33 patients diagnosed with INH and 33 age- and sex-matched controls, children without any liver disease, were recruited. Baseline serum total bilirubin (TB), direct bilirubin (DB), and alkaline phosphatase (ALP) of the patients were obtained from their medical records. Genotypes for CD14/-159 and TNFα/-308 were determined via PCR and direct sequencing.

Results. No significant difference was seen between the frequency of the CD14/-159 T allele (p=0.86) nor the TNFα/-308 A allele (p=0.62) between INH patients and controls. There was also no significant difference between the genotypic distribution of the INH and control populations for both CD14/-159 (p=0.54) and TNFα/-308 (p=0.62). There were also no significant differences noted between the different genotypes of CD14/-159 and TNFα/-308 and levels of alkaline phosphatase (p=0.65, p=0.91), total bilirubin (p=0.89, p=0.75), and direct bilirubin (p=0.93, p=0.68).

Conclusion. In this preliminary study, CD14/-159 and TNFα/-308 showed no association with the development of INH among Filipinos.

Key Words: Idiopathic neonatal hepatitis, CD14, TNFα, promoter polymorphism, Filipino

Introduction

Cholestasis, a condition in which bile flow from the liver is impaired, may be caused by two events - a problem in bile formation of hepatocytes, and the obstruction of bile flow through the biliary trees[1] both of which lead to a buildup of bile substances in the blood. Bile is produced through bile salt uptake of hepatocytes from the blood, followed by excretion of bile salts into the biliary canaliculus. These processes involve various membrane transporters found on both the basolateral and the canalicular aspects of hepatocytes.[2] Cholestatic jaundice affects approximately 1 in every 2,500 infants worldwide with biliary atresia and idiopathic neonatal hepatitis (INH) as the most common causes.[3]

Idiopathic neonatal hepatitis (INH) has an incidence of 1 in 4,800 to 9,000 live births in North America.[4] A systematic review done by Gottesman, et al. estimates INH to comprise 26.0% of cases of conjugated hyperbilirubinemia in infancy. However, because of recent developments in determining possible causes of INH, the disorder now only partakes 15% of neonatal cholestasis cases.[5] In parts of Asia, majority of neonatal cholestasis cases are still idiopathic. In Malaysia, INH comprises 38% of their neonatal cholestasis cases[6] while in Thailand 23% of these cases were idiopathic.[7] In a five-year period (2006-2010), the Philippine General Hospital had 231 cases of INH, where giant cell hepatitis was proven through percutaneous liver biopsy.[8]

Being a diagnosis of exclusion, a patient is said to have INH after obtaining negative results in workups for other etiologies – ultrasound of the hepatobiliary tree, metabolic newborn screening and determination of any evidence of infection such as toxoplasmosis, rubella, cytomegalovirus, herpes and hepatitis B virus. Measurement of liver function tests is done including direct bilirubin (DB), indirect bilirubin (IB), and alkaline phosphatase (ALP) as a measure of cholestasis and transaminases to determine degree of hepatocellular damage. A patient with INH clinically presents with jaundice, hepatosplenomegaly, elevated serum bilirubin and transaminases. Histopathologic features of INH include lobular disarray due to swelling of hepatocytes, focal hepatic necrosis, giant cell transformation, and extramedullary hematopoiesis.[2]

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The etiology of INH is still unknown. Since 1970, possible causes of INH which include genetic and different metabolic disorders have been determined. This led to an approximately 50% decrease in the percentage of infants with neonatal cholestasis diagnosed with the disease. Despite the developments on the topic, a small number of patients were found not to have genetic defects, thereby, necessitating further research in order to determine the etiology of this disease.[9]

Recently, a study was published regarding the association of the CD14/-159 and TNFα/-308 promoter polymorphism to the development of INH[10] and showed that there is a possible association between CD14/- 159 polymorphism and the development of INH.

The CD14 gene encodes a surface antigen that is mainly expressed on monocytes. The protein plays a role in the mediation of the innate immune response to lipopolysaccharide, an antigen produced by gram-negative bacteria.[11] This suggests a possible etiology in which processes downstream of CD14 expression may be implicated in the development of INH.

The TNFα gene, on the other hand, is located in the Major Histocompatibility Complex Class 3 region on chromosome 6.[12,13] TNFα is a pro-inflammatory cytokine produced by Kupffer cells upon activation by various stimuli. A common stimulus is lipopolysaccharide, during hepatic clearance of infection. TNFα release leads to a downregulation of certain membrane transporters; thereby, causing a decrease in bile flow, ultimately leading to bile flow cholestasis.[14] The TNFα promoter polymorphism G(-308)A has been found to be associated with higher TNFα production,[15] as well as various hepatobiliary conditions including faster clearance of HBV infection, allograft rejection in the liver, primary sclerosing cholangitis,[10] chronic HCV infection[15] with severe inflammation, and fibrosis in non-alcoholic fatty liver disease.[16]

This study, the first to be done on INH among Filipino patients, determined the association of TNFα/-308 and CD14/-159 in the development of idiopathic neonatal hepatitis among Filipino children.

Methods

Study Population

As a preliminary study only, thirty-three Filipino children (16 males and 17 females) histologically diagnosed with INH at the Section of Gastroenterology, Hepatology and Nutrition of the Department of Pediatrics, University of the Philippines Manila – Philippine General Hospital were included in this study. Exclusion criteria were children diagnosed with neonatal cholestasis with an identified etiology, no informed consent, and those who had no liver biopsy. Thirty-three age- and sex-matched children (16 males and 17 females) without any history of jaundice or other liver conditions, having their laboratories taken at the Department of Laboratories, UP-PGH were recruited as controls.

Polymorphism Analysis

Genomic DNA was extracted from peripheral blood of both patients and controls using the QIAmp DNA Mini/Midi Kit (Valencia, CA, USA). A 347bp fragment with the single nucleotide promoter polymorphism TNF-α -308 was amplified using the primers 5′ – CTCAGGACTCAACACAGC – 3′ and 5′ – TCGGTTTCTTCTCCATCG – 3′. The primers used to amplify the 295kb fragment containing the CD14 C(-159)T polymorphism were 5′ – ATCATCCTTTTCCCACACC – 3′ and 5′ – AACTCTTCGGCTGCCTCT – 3′.

Each PCR reaction mixture for both CD14/-159 and TNFα/-308 contained 1 uL of 10x PCR buffer, 0.3 uL of 50 mM MgCl2, 0.2 uL of 10 mM dNTP, 0.2 uL of 10 uM CD14 or TNFα forward primer, 0.2 uL of 10 uM CD14 or TNFα reverse primer and 0.2 uL of 50x TiTaq (1U TiTaq), 100 ng of genomic DNA diluted to 10uL with distilled, deionized water.

The amplification profile for CD14/-159 is as follows: initial denaturation (7 mins, 95°C), 35 cycles of denaturation (30s, 95°C), annealing (30s, 62.5°C), and extension (30s, 72°C), followed by final extension (10 mins, 72°C) and for TNFα/-308: initial denaturation (7 mins, 94°C), 35 cycles of denaturation (30s, 94°C), annealing (30s, 59.7°C), and extension (30s, 72°C), followed by final extension (10 mins, 72°C).

All PCR products, stained with GelRed™ were run on 2% agarose gels and visualized using UV transillumination. The amplicons were subsequently directly sequenced using the ABI 3730XL DNA ANALYZER (Applied Biosystems, USA).

Biochemical Characteristics

Baseline serum levels of alkaline phosphatase, total bilirubin, direct and indirect bilirubin were obtained from the patient's medical records. These blood tests were done at the Department of Laboratories of the Philippine General Hospital using standard techniques.

Statistical Analysis

Allele and genotype frequencies were estimated by the gene counting method. Odds ratios together with 95% confidence intervals were calculated using the χ^2 test of association and the Hardy-Weinberg equilibrium was tested for each SNP using the χ^2 test.

Mean and standard deviations of alkaline phosphatase and conjugated bilirubin levels were computed from the information collected from the patients. ANOVA was used to determine if there was a significant difference between the biochemical characteristics of patients with different genotypes.

A p-value of less than 0.05 was considered statistically significant for all statistical tests performed. All statistical tests were performed using OpenEpi v3.0.

Ethical Considerations

The protocol underwent review and approval by the Research Implementation and Development Office and the University of the Philippines Manila - Research Ethics Board (UPM-REB). Written informed consents were obtained from the parents or the legal guardians prior to inclusion of the participants in the study.

Results

Study Participants

A total of 33 cases and 33 controls were recruited for the study (Table 1). The age of the participants recruited ranged from 1 month to 13 months of age. There was no significant difference (p=0.86) between the ages of the case (3.18±1.96 months) and control (3.27±2.31 months) groups. The study population was composed of 16 pairs of male and 17 pairs of female participants.

Table 1. Demographic Characteristics of the Study Population

	INH (n=33)	Control (n=33)	p-value (t-test)
Age (months)	3.18±1.96	3.27±2.31	0.86
Sex (M/F)	16/17	16/17	

Allele and Genotype Frequency among INH Patients and Controls

PCR amplicons (347bp TNFα fragment and 295 bp CD14 fragment) were sequenced to determine the presence of the TNFα and CD14 promoter polymorphisms in all study participants. Thirty-three (33) age- and sex-matched INH cases and controls were analyzed for TNFα and CD14, respectively.

There was no significant difference between the allelic distribution of the A and G allele for TNFα/-308 (p=0.62) and the C and T allele for CD14/-159 (p=0.86) for INH cases and controls (Table 2).

TNFα/-308 A/A genotype was absent among INH patients and controls. Using the dominant model (A/A + G/A vs G/G), there was no significant difference between the G/G genotype of INH patients and controls (p=0.62). Using a recessive model (T/T vs C/T + C/C), there was no significant difference between the T/T genotype of INH patients and controls (p=0.54). The results show that neither the TNFα/-308 nor the CD14/-159 promoter polymorphisms confer an increased risk for developing INH in the Filipino population.

Biochemical Characteristics of INH Patients and their Relationship to CD14/-159 and TNFα/-308 Genotype

There was no significant difference between the levels of alkaline phosphatase, total bilirubin and direct bilirubin among the different genotypes (Table 3).

Discussion

No study has so far been conducted regarding the possible role of genes in idiopathic neonatal hepatitis (INH) here in the Philippines.

A study conducted by Shih and colleagues has shown the association of CD14/-159 promoter polymorphism with biliary atresia and INH in Taiwanese children.[1] In the same study, it was found that TNFα/-308 promoter polymorphism was not associated with the said diseases. However, as shown in Table 2, this does not hold true for the Philippine population; both the CD14/-159 (C>T) and TNFα/-308 (G>A) polymorphisms were not associated with the development of INH in neonates in Filipinos.

TNF alpha and Liver Diseases

TNFα is a cytokine secreted by macrophages and cytotoxic T lymphocytes in the liver. It has pro-inflammatory and antiviral properties that give it a major role in hepatic injury and fibrosis.[17]

Other TNFα gene SNPs associated to liver diseases, such as hepatitis B virus infection, are -1031T>C, -863C>A,

Table 2. TNFα -308 and CD14-159 Polymorphism Among Patients with INH and Controls

	INH (%) (n=33)	Control (%) (n=33)	Odds Ratio (95% CI)	p-value (2-tail)
CD14 C(-159)T Polymorphism				
Allele				
C	38 (57.58%)	37 (56.06%)	0.94 (0.47, 1.87)	0.86
T	28 (42.42%)	29 (43.94%)		
Genotype				
C/C	12 (36.36%)	9 (27.0%)		
C/T	14 (42.42%)	19 (57.58%)		
T/T	7 (21.21%)	5 (15.0%)	1.51 (0.43, 5.35)	0.54
TNFα G(-308)A Polymorphism				
Allele				
A	2 (3.03%)	1 (1.51%)	2.03 (0.18, 22.96)	0.62
G	64 (96.97%)	65 (98.48%)		
Genotype				
A/A	0	0		
A/G	2 (6%)	1 (3.03%)		
G/G	31 (93.94%)	32 (96.97%)	2.07 (0.18, 23.94)	0.62

Table 3. Biochemical Characteristics of INH Patients According to TNFα -308 and CD 14 -159 Genotypes

	TNFα -308 Polymorphism			CD14 C(-159)T Polymorphism			
	G/A (n=2)	G/G (n=31)	p-value	T/T (n=7)	C/T (n=14)	C/C (n=12)	p-value
Alkaline Phosphatase (IU/L)	509±140	536.38±320.96	0.91	629.64±448.49	535.05±183.88	480.56±400.12	0.65
Total Bilirubin (mg/dL)	10.44±2.18	11.03±4.39	0.75	11.70±3.75	10.77±3.94	10.86±5.17	0.89
Direct Bilirubin (mg/dL)	7.67±0.89	6.71±3.21	0.68	7.16±2.57	6.69±3.08	6.62±3.67	0.93

-857C>T and -238G>A.[12,18] It is possible that other TNFα SNPs besides the studied -308G>A are implicated in INH. These SNPs can be studied in the future to determine possible associations with INH in the Filipino population.

A meta-analysis by Chen et al. revealed that the TNFα/-308 polymorphism is not associated with the development among Asian populations. Their results showed that among Asians, the homozygous A/A and the heterozygous G/A alleles were the most common.[19] This data supports the results of this study, wherein participants are all Filipinos and are from the Asian population.

Other contributory factors, including the role of other cytokines should also be taken into consideration. Bader El Din, et al. showed that TGFB1/-159 polymorphism had a synergistic effect to TNFα/-308 in hepatic fibrosis progression.[20] Hence, there is a need to analyze multiple polymorphisms and the role of other inflammatory factors in the development of INH.

CD14 and Liver Diseases

The CD14/ -159 polymorphism has been associated with many diseases such as myocardial infarction,[21] alcoholic liver disease[22] and other atopic diseases.[23] The CD14 gene expresses a protein which is significant for lipopolysaccharide-dependent signal transduction[24] and thus, the general hypothesis is that the subsequent immune responses generated through the endotoxin receptor CD14 plays a role in the development of the different diseases.

The CD14/ -159 polymorphism affects expression of the endotoxin receptor, with the T allele generating a higher expression of the receptor because of decreased affinity of an inhibitory factor for transcription (Sp3) to the regulatory region.[25] It is expected then, that an individual with a T allele would generate a more potent immune response through the CD14 receptor pathway as compared to an individual with a C allele because of the higher expression of the said protein. Thus, the more potent inflammatory response contributes to the pathology of different diseases.

Different studies have also shown conflicting results with regard to this hypothesis. While some studies have shown the association of the TT genotype with elevated sCD14 levels and cirrhosis,[26] the study conducted by Shih et al. showed that those carrying the T allele developed cirrhosis but had lower sCD14 levels.

Other studies regarding CD14/ -159 polymorphism and IgE circulation have also been conducted. However, studies associating the CD14/ -159 polymorphism with IgE circulation have also produced conflicting results; some have shown the association of the CC genotype with elevated IgE levels,[27] while others have shown otherwise.[28-29] The discrepancies thus lead us to question the exact role of the CD14/ -159 polymorphism in the development of different diseases especially in inflammation. It may be that CD14 is only one of the many different factors that affect the outcome of these diseases.

To explain the differences, Vercelli suggests that there exists an "endotoxin switch" wherein differences in the environmental exposure to endotoxin may have an effect on the modulation of the polymorphism; that exposure to different antigen concentrations would result in opposite profiles of Th differentiation.[30] In his model, when a population is exposed to very low and very high endotoxin loads, Th responses would be similar across the population and genetic differences would have little contribution to these responses. Polymorphisms only come into play within a narrow range of endotoxin load. These populations which are exposed to intermediate levels of endotoxin are more sensitive to small variations in CD14 expression.[30]

This hypothesis was confirmed by a study of Eder et al. Their study showed that at different levels of exposure, CD14/ -159T polymorphism would have different effects. They found out that CD14/ -159T is neutral when tested for association with serum IgE levels in the totality of the population when exposure is not taken into account. At intermediate levels of exposure, the polymorphism is protective against increased IgE while at high exposure, it is associated with high IgE levels.[31] This may then explain the differing results of this study to the study done by Shih et al. which was done in Taiwanese children consulting at Kaohsiung Chang Gung Memorial Hospital. The environmental exposures of the Filipino children studied may differ from the environmental factors of the Taiwanese children. Hence, it is also important to keep in mind that diseases are not merely the product of genetics but also a product of epigenetics and other factors, including environmental exposures that may alter genetic expression.

The discrepancies may also be accounted for by the different disease entities between the studies. The etiology of INH in the Philippines may differ from the etiology of INH in other countries since INH may actually be regarded as a hodgepodge of different diseases.[9]

Biochemical Parameters and Genotypic Distribution in INH Cases

Total bilirubin, direct bilirubin and alkaline phosphatase levels of INH patients with the different

genotypes were compared. The data generated suggests that both the CD14/ -159 polymorphism and the TNFα/ -308 do not have any association with serum levels of these markers.

Mean levels of DB and ALP across different TNFα/ -308 genotypes among the group of INH cases did not vary significantly in this study. A research done by Xia et al. in 2011 on HBV infection showed that patients with TNFα/ -308 G/G genotype produced lower serum TNF-alpha than patients with the A/G and the A/A genotypes.[13] A study on leptospiral hepatitis by Rizvi et al. in 2011 measured the mean ALT, AST and ALP levels together with TNFα levels in the serum. Though their patients had low TNFα levels (45.63pg/mL) in the blood, they observed elevated levels of ALT, AST and ALP.[32]

Compared to the findings of the aforementioned studies, the results of this research are contrasting. Majority of the INH cases had the G/G genotype, however, no significant difference was observed in the mean ALP levels of the groups with G/A genotype. However, a possible explanation for this is the low frequency of the G/A genotype in the sample of INH cases for this study. Conclusive comparisons between DB and ALP levels across different genotypes cannot be made.

A study by Poullis et al. showed that there was an association between CD14/-159 and liver enzyme levels in 310 British adults. In particular, CD14/-159 TT homozygotes had reduced levels of alanine aminotransferase, aspartate aminotransferase and gamma-glutamyl transpeptidase.[33] However, a similar study conducted by Gonzalez-Quintela in the Spanish population showed that there was no such association and that CD14/-159 TT even had the highest serum gamma-glutamyl transpeptidase levels.[34] Our results cannot conclude if there is a direct role of the CD14/-159 in the elevation of liver enzymes.

Since no association was found between the CD14 -159 promoter polymorphism and alkaline phosphatase and bilirubin levels, it may be suggested that CD14 expression does not play a direct role in the elevation of these markers.

Conclusion

Results of this preliminary study showed that the CD14/ -159 and the TNFα/ -308 polymorphisms do not have an association with the development of idiopathic neonatal hepatitis among Filipinos. There was no significant difference between the frequencies of A and G allele for TNFα/-308 and C and T allele for CD14/-159 for INH cases and controls. Lastly, there was no significant difference between the levels of alkaline phosphatase, total bilirubin, and direct bilirubin among the different genotypes for both polymorphisms.

The small population size of our study may have affected the outcome. Increasing the sample size would reflect data that is closer to the population mean and might uncover a significant difference in the biochemical markers between the three genotypes. For future studies, other factors that may affect gene expression may also be taken into consideration; this would include considering the synergistic effects of multiple polymorphisms thereby the need to analyze multiple polymorphisms. A more extensive study which include the maternal and prenatal history and other exposures of INH patients may also be done so as to take into account other external factors that may be associated with the development of INH, in concordance with their CD14 -159 SNP.

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Statement of Authorship

Florence Giannina F. San Juan and Annavi Marie G. Villanueva contributed equally to this paper (Joint first authorship). All authors have approved the final version submitted.

Author Disclosure

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