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Pathogenic variants in TIRAP and MyD88 are not associated with neonatal sepsis in a South Indian population.

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Abstract

Genetic variants and their association with sepsis are the futuristic consideration for the development of potential prognostic markers. Polymorphisms in Toll-interleukin-1 receptor *(TIRAP)* and Myeloid differentiation primary response gene 88 (*MyD88)* were found to be significantly associated with infectious diseases like tuberculosis and malaria. In this study, we determined the association of these pathogenic variants in neonatal sepsis. We enrolled 110 newborns with sepsis as cases and 117 newborns without sepsis as controls from Dravidian population from Tamilnadu, India. The genotypic and allelic frequencies of the variants in *TIRAP* (rs8177374) and *MyD88* (rs6853) were studied TaqMan Genotyping assay kits. The plasma levels of C-reactive protein (CRP) and Tumor necrosis factor-alpha (TNF-α) were estimated using commercially available ELISA kits. The genotypic and allelic frequencies of both the variants studied were found to be not associated with susceptibility to neonatal sepsis. However, the genotype AA was significantly associated with mechanical ventilation and high plasma CRP levels ($P < 0.05$) among the population studied. This is probably the first study to determine the genetic associations of neonatal sepsis among Indian population. No significant association of the pathogenic genetic variants in *TIRAP* and *MyD88* were found in our study.

Keywords: Neonatal sepsis, Genetic associations, *TIRAP*, *MyD88*

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Introduction

Neonatal sepsis remains the biggest burden in pediatric healthcare due to its high mortality rate. Apart from the infection and environmental factors, genetic predisposition also plays a vital role in host immune response during sepsis. Several genetic association studies had been done in sepsis mainly targeting the adults [1-2] and hardly any data is available on neonatal sepsis. Moreover, the available data is from western populations and till date no genetic association studies on neonatal sepsis had been published in Indian population, this is probably the first study.

The genetic variants in innate immune modulator genes like Toll-like receptor 4 (*TLR4*), Toll-like receptor 2 (*TLR2*), cluster of differentiation 14 (*CD14*) had been studied for their significant role in sepsis risk [3-5]. The genetic association studies are purely population specific [6]. Hence, before concluding the genetic risk of sepsis, the genetic association data should be available in all contemporary ethnic groups, from Chinese Han to Russians, from Dravidians to Germans.

In this study, we have analyzed two Single nucleotide polymorphisms (SNPs), rs6853 and rs8177374 present in the genes Myeloid differentiation primary response gene 88 (*MyD88*) and Toll-interleukin 1 receptor (*TIRAP*) respectively. These two genes were found to play significant roles in immunological signaling pathways occurring during sepsis [7-9]. The SNP rs6853 (A/G) in *MyD88* is present in 3' untranslated region and rs8177374 (C/T) is a missense variant in *TIRAP* that induces an aminoacid change (S180L). Both the variants were found to be significantly associated with infections like tuberculosis [10]. In the present study, we attempted to elucidate the association of these two SNPs with risk and outcome of neonatal sepsis.

Material and Methods

This study was conducted in the Neonatology Division of our tertiary care referral hospital during the period of June, 2013 to April, 2014. The study was approved by Institute scientific advisory, human ethics committees and registered with Clinical Trials Registry of India (CTRI/2012/09/002987).

A total of 110 newborns as cases and 117 newborns as controls belonging to Dravidian population from Tamilnadu, India were enrolled in this study. Babies of age less than 28 days with clinical characteristics suggestive of sepsis and at least two positive out of the three screening tests, micro Erythrocyte Sedimentation Rate, mESR (> Age in days+3mm/hr), CRP (>1 mg/dL), band cell count (>20%) were enrolled as cases. Newborns without signs of sepsis and blood sampled for minor complaints and high risk newborns with septic screening tests negative were enrolled as controls. Babies with surgical conditions, major congenital defects, maternal record of infections/inflammations and Apgar score <6/10 at 5mt were excluded.

Genotype Analysis

Peripheral venous blood sample (500 µL) was collected in an Ethylene diamine tetraacetic acid (EDTA) vacutainer from the newborns, after getting written informed consent from their parents. Genomic DNA was isolated from the blood samples using Qiagen Blood DNA Mini kit (Qiagen, Hilden, Germany) following standard protocol. The extracted genomic DNA was checked for quantity and quality using Nanodrop 2000 spectrophotometer (Thermo scientific, USA).

Polymerase chain reaction (PCR) was carried out in Rotor Gene Q real-time PCR (Qiagen, Hlden, Germany) with functionally tested TaqMan Genotyping Assay kits (Invitrogen, CA, USA). The assay id for the SNP rs6853 is C_8824617_10 and for rs8177374 is C_25983622_10. For each SNP, PCR was performed in 20 µL volume containing 10 µL of TaqMan Genotyping master mix (Applied Biosystems, California, USA), 1 µL of 100 ng genomic DNA, 1 µL of TaqMan genotyping assay mix and 8 µL of nuclease free water (Promega, Wisconsin, USA).

The PCR conditions for both SNPs were an initial denaturation at 95^0C for 10 min; followed by 40 amplification cycles of 95^0C for 15 sec and annealing/extension at 60^0C for 1 min. Fluorescence acquisition was done at the end of each cycle following gain optimization for both VIC and FAM dyes in TaqMan genotyping assay mixture. Data analysis was carried out using the software Rotor-Gene Q-Pure Detection v2.1.0 (Build 9). Allelic discrimination was done based on Ct values and scatter plot analysis.

The concentration of CRP and TNF-α in plasma were estimated using sandwich Enzyme-linked immunosorbent assay (ELISA) kits (DBC, Canada and RayBiotech, Georgia, USA respectively).

Plasma concentration of C - reactive protein (CRP) was significantly different among cases and controls (Cases: 1.1 ± 0.1 mg/dL; Controls: 0.7 ± 0.4 mg/dL). No significant difference was found in plasma Tumor necrosis factor - alpha (TNF-α) level. The plasma level of CRP was significantly associated with the SNP rs6853. The newborns with genotype AG had low CRP concentrations (0.95 ± 0.07 mg/dL) and AA genotype had higher concentration (1.13 ± 0.12 mg/dL). No genotypes in rs8177374 were associated with CRP and TNF-α concentration.

Statistical Analysis

All categorical variables were given as percentages and frequencies. Continuous variables were represented as Mean±SD. The Hardy-Weinberg equilibrium was verified using Chi square (χ^2) test. χ^2 test was used to compare the genotype and allele frequency of the SNPs among cases and controls. The relation of plasma concentrations of CRP and TNF-α with genotype and allele frequencies among cases and controls were assessed using Independent student's t-test. A two sided P-value <0.05 was considered significant. All statistical analysis was done in SPSS v16 (SPSS Inc., Chicago, IL, USA) and Microsoft Excel, 2007.

Results

The baseline characteristics of newborns with sepsis and without sepsis are given in Table 1. The cases and controls were homogenous for baseline characteristics and sepsis risk factors like premature rupture of membrane (PROM). The mean gestational age in cases and controls were 35.14±3.5 and 37.18±3.4 weeks respectively.

The clinical outcome of babies with sepsis is given in Table 2. Out of 110 newborns in septic group, 75 cases had positive blood culture. Among the Gram negative bacteria, *Klebsiella pneumoniae, Escherichia coli* were most common. The only gram positive bacterium isolated was *Staphylococcus aureus.* Fungal species, *Candida non-albicans* and *Candida glabrata* were also isolated. The sepsis outcomes like Disseminated intravascular coagulation (DIC), shock, organ failure were assessed following standard protocols like Sequential organ failure assessment score (SOFA).

The study population was found to be in Hardy-Weinberg equilibrium. Genotype analysis showed that the two SNPs rs6853 in *MyD88* and rs8177374 in *TIRAP* were not associated with neonatal sepsis. The genotype frequencies along with odds ratio are given in Table 3. Haplotype analysis also failed to show significant association with neonatal sepsis. The allele frequencies of the two SNPs were compared with the allele frequency of healthy individuals genotyped in HapMap project (Table 4).

The genotype and allele frequencies were analyzed for their association with sepsis outcomes. No significant association was found with respect to the type of infection, mortality or other outcomes, except the association of genotype AA in rs6853 with mechanical ventilation requirement [Odds Ratio (OR) - 0.15 (0.02 - 1.18); $P < 0.05$].

Table 1. *Baseline characteristics of the subjects enrolled in this study.*

Parameters		Cases, ***n*=110** *n* (%)	Controls, ***n*=117** *n* (%)	***P* value**
Gender	Male	61 (55.5)	64 (54.7)	0.90
	Female	49 (44.5)	53 (45.3)	
Mode of Delivery	Spontaneous vaginal	57 (51.8)	52 (44.4)	0.56
	Caesarian	32 (29)	38 (32.5)	
	Operative vaginal	21 (19.2)	27 (23.1)	
Maturity	Preterm	58 (52.7)	50(42.7)	0.15
	Term	52 (47.3)	67 (57.3)	
Birth Weight	Low birth weight	67 (60.9)	59 (50.4)	0.13
	Normal	43 (39.1)	58 (49.6)	
	Mean ± SD (gms)	2386.03 ± 666	2429.23 ± 733	
APGAR at 5’	Median	8 (7,9)	8 (7,9)	1.00
Gravida	G1	73 (66.4)	69(59)	0.27
	≥ G2	37 (33.6)	48 (41)	
Liquor Quality	Meconium stained	23 (21)	36 (30.7)	0.11
	Clear	87 (79)	81 (69.3)	
Rupture of Membrane	PROM[a]	28 (25.5)	38 (32.5)	0.27
	Normal	82 (74.5)	79 (67.5)	

[a]*Premature rupture of membranes*

Table 2. *Clinical outcomes in the newborns with sepsis.*

Outcomes	**Sepsis Cases, *n*=110** *n* (%)
Positive blood culture	75 (68.2)
Gram negative	51 (68)
Gram Positive	2 (2.6)
Fungi	10 (13.4)
Mixed infection	12 (16)
Expired	27 (24.5)
Respiratory Distress Syndrome	63 (57.3)
Shock	32 (29.1)
Disseminated Intravascular Coagulation	32 (29.1)
Requirement of mechanical ventilation	37 (33.6)
Seizures	22 (20)
Pneumonia	12 (10.1)
Meningitis	5 (4.5)
Hypoglycemia	11 (10)
Generalized Edema	11 (10)
Thrombocytopenia	66 (60)
Liver failure	16 (14.5)
Renal failure	11 (10)
Number of days in ICU[a] (Mean ± SD)	16.8±9.02

[a]*Intensive care unit*

Table 3. *Genotype frequencies among cases and controls.*

SNP	**Gene**	**Genotype**	**Cases, *n(%)***	**Controls, *n(%)***	**OR (95% CI)**	**Chi**	**P**
rs6853	*MyD88*	GG	0 (0)	0 (0)	1.36 (0.52-3.59)	0.39	0.53
		AG	10 (9)	8 (6.8)			
		AA	100 (91)	109 (93.2)			
rs8177374	*TIRAP*	CC	74 (67.3)	77 (65.8)	1.07 (0.61-1.85)	0.05	0.81
		CT	32 (29.1)	36 (30.8)			
		TT	4 (3.6)	4 (3.4)			

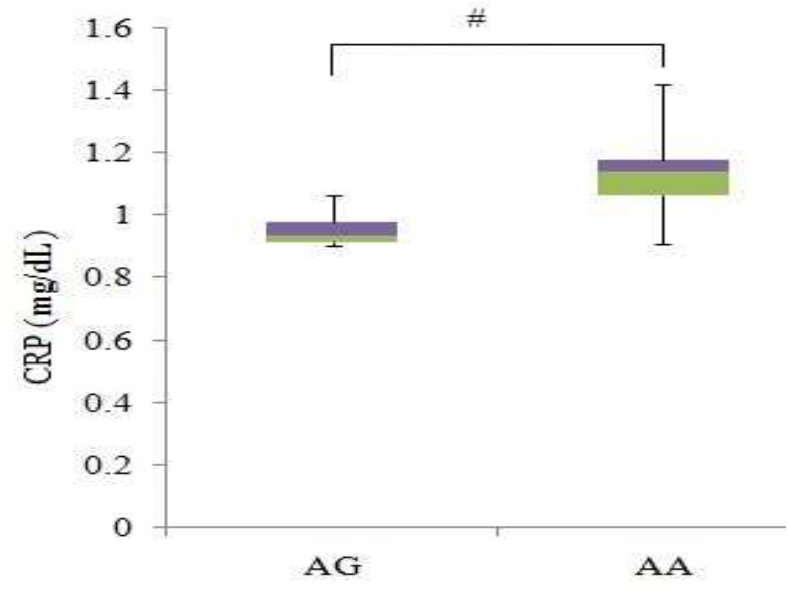

Figure . *Higher CRP concentration seen in AA genotype compared to AG in MyD88. ($^{\#}P < 0.03$)*

Table 4. *Allele frequencies in South Indian population compared to HapMap populations (All frequencies are in percentages).*

SNP	Alleles	SI - TSD[a]		CEU[b]	CHB[c]	JPT[d]	YRI[e]	ASW[f]	GIH[g]	LWK[h]	MEX[i]	MKK[j]	TSI[k]
		Cases	Controls										
rs6853	G	4.7	3.4	11.5	4.9	0.6	33.2	27.6	6.2	28.9	5	38.8	18.2
	A	95.3	96.6	88.5	95.1	99.4	66.8	72.4	93.8	71.1	95	61.2	81.8
rs8177374	C	81.8	81.2	83	98.8	97	100	94.9	86.9	99.4	90	95.4	79.9
	T	18.2	18.8	17	1.2	3	0	5.1	13.1	0.6	10	4.6	20.1

[a]Dravidian population from Tamilnadu, India; [b]Utah residents with Northern and Western European ancestry from the CEPH collection; [c]Han Chinese from Beijing, China; [d]Japanese from Tokyo, Japan; [e]Yoruba trios from Ibadan, Nigeria; [f]African ancestry in Southwest USA; [g]Gujarati Indians in Houston, Texas; [h]Luhya in Webuye, Kenya; [i]Mexican ancestry in Los Angeles, California; [j]Maasai in Kinyawa, Kenya; [k]Tuscans in Italy

Discussion

The genetic association studies in neonatal sepsis are sparse among Asian countries. Some of the significant genetic variations reported to be associated with sepsis in western countries were found not associated with sepsis in Asian countries [11]. This may be due to the vast genetic diversity among western population and Asian population.

The two SNPs analyzed in this study were reported previously to be pathogenic in serious infections like tuberculosis, malaria [10] and bacteremia [12] among the European population. Khor et al., published a remarkable study on genetic association of rs8177374 with tuberculosis, malaria and bacteremia. They found significant association of *TIRAP* S180L among the populations of Gambia, Kenya, United Kingdom and Vietnam. They also showed that impairment of TLR2 signaling pathway by *TIRAP* S180L [13]. In contrast, the studies done in Asian population showed no significant association of this genetic variation with sepsis [14,15].

We conducted this study since there is no data available regarding the association of rs6853 and rs8177374 with neonatal sepsis among the Indian population. The genetic diversity of Indian population is highly complex in nature which is yet to be explored [16]. Recent evidences of gene flow from east and west Eurasia has been reported [17]. The data of human genetic variants retrieved from huge projects like HapMap are not adequate to study this complex population. More than 40 different ethnic groups of people live in India. Only with huge number of ethnicity specific genetic association studies, the genetic

risks of Indian population towards various diseases can be explored.

In this study, we found no significant association of the pathogenic SNPs rs6853 and rs8177374 with neonatal sepsis. Both genotype and allele frequencies failed to yield an association. The previous studies reported were in adult sepsis and no studies had been done in neonatal age group around the globe. The allele frequencies found in our study were comparable to the normal allele frequency in closely related populations like Chinese Han and Japanese.

The AA genotype in rs6853 was significantly associated with plasma CRP level, which can be inferred for its association with disease severity as CRP increases with inflammation [18]. The same can be evidenced by the significant association of genotype AA in rs6853 with mechanical ventilation requirement. Animal experiments showed that expression of *MyD88* and *TIRAP* was indispensable in bronchoconstriction and acute inflammatory lung pathology to endotoxin [19]. Also serum CRP was found elevated in *Haemophilus influenza* infection leading to worse lung function [20]. Unfortunately, no studies are available to explain the role of these genetic variants in pulmonary diseases, so further insights are required in this context.

In conclusion, no significant association of *MyD88* and *TIRAP* variants were found with neonatal sepsis in Dravidian population from Tamilnadu, in contrast to previous reports from western populations. This contradicting result can be further verified with large sample size to arrive at a clear picture.

References

1. Mansur A, von Gruben L, Popov AF, Steinau M, Bergmann I, Ross D, et al. The regulatory toll-like receptor 4 genetic polymorphism rs11536889 is associated with renal, coagulation and hepatic organ failure in sepsis patients. J Transl Med 2014; 12(1): 177-85.
2. Manocha S, Russell JA, Sutherland AM, Wattanathum A, Walley KR. Fibrinogen-beta gene haplotype is associated with mortality in sepsis. J Infect 2007; 54(6): 572-7.
3. Bronkhorst MW, Boyé ND, Lomax MA, Vossen RH, Bakker J, Patka P, et al. Single-nucleotide polymorphisms in the Toll-like receptor pathway increase susceptibility to infections in severely injured trauma patients. J Trauma Acute Care Surg 2013; 74(3): 862-70.
4. Zhang AQ, Yue CL, Gu W, Du J, Wang HY, Jiang J. Association between CD14 promoter -159C/T polymorphism and the risk of sepsis and mortality: a systematic review and meta-analysis. PLoS One 2013; 8(8): e71237.
5. Nachtigall I, Tamarkin A, Tafelski S, Weimann A, Rothbart A, Heim S, et al. Polymorphisms of the toll-like receptor 2 and 4 genes are associated with faster progression and a more severe course of sepsis in critically ill patients. J Int Med Res 2014; 42(1): 93-110.
6. Zhu L, Li X, Miao C. Lack of association between TLR4 Asp299Gly and Thr399Ile polymorphisms and sepsis susceptibility: a meta-analysis. Gene 2012; 501(2): 213-8.
7. Bhinder G, Stahl M, Sham HP, Crowley SM, Morampudi V, Dalwadi U, et al. Intestinal epithelial specific MyD88 signaling impacts host susceptibility to infectious colitis by promoting protective goblet cell and antimicrobial responses. Infect Immun 2014. pii: IAI.02045-14.
8. Wang J, Dong B, Tan Y, Yu S, Bao YX. A study on the immunomodulation of polysaccharopeptide through the TLR4-TIRAP/MAL-MyD88 signaling pathway in PBMCs from breast cancer patients. Immunopharmacol Immunotoxicol 2013; 35(4): 497-504.
9. Stokes JA, Corr M, Yaksh TL. Spinal toll-like receptor signaling and nociceptive processing: regulatory balance between TIRAP and TRIF cascades mediated by TNF and IFNβ. Pain 2013; 154(5): 733-742.
10. Capparelli R, De Chiara F, Di Matteo A, Medaglia C, Iannelli D. The MyD88 rs6853 and TIRAP rs8177374 polymorphic sites are associated with resistance to human pulmonary tuberculosis. Genes Immun 2013; 14(8): 504-511.
11. Fang Y, Zhang L, Zhou G, Wang Z, Feng K, Lou Z, et al. TRAF6 polymorphisms not associated with the susceptibility to and severity of sepsis in a Chinese population. World J Emerg Med 2010; 1(3): 169-75.
12. Kumpf O, Giamarellos-Bourboulis EJ, Koch A, Hamann L, Mouktaroudi M, Oh DY, et al. Influence of genetic variations in TLR4 and TIRAP/Mal on the course of sepsis and pneumonia and cytokine release: an observational study in three cohorts. Crit Care 2010; 14(3): R103.
13. Khor CC, Chapman SJ, Vannberg FO, Dunne A, Murphy C, Ling EY, et al. A Mal functional variant is associated with protection against invasive pneumococcal disease, bacteremia, malaria and tuberculosis. Nat Genet 2007; 39(4): 523-528.
14. Song Z, Yin J, Yao C, Sun Z, Shao M, Zhang Y, et al. Variants in the Toll-interacting protein gene are associated with susceptibility to sepsis in the Chinese Han population. Crit Care 2011; 15(1): R12.
15. Song Z, Tong C, Sun Z, Shen Y, Yao C, Jiang J, et al. Genetic variants in the TIRAP gene are associated with increased risk of sepsis-associated acute lung injury. BMC Med Genet 2010; 11: 168.
16. Xing J, Watkins WS, Hu Y, Huff CD, Sabo A, Muzny DM, et al. Genetic diversity in India and the inference of Eurasian population expansion. Genome Biol 2010; 11(11): R113.

17. Tamang R, Singh L, Thangaraj K. Complex genetic origin of Indian populations and its implications. J Biosci 2012; 37(5): 911-9.
18. Vladimirova SG, Tarasova LN, Sokol'skaia OIu, Cherepanova VV. C-reactive protein as a marker of the severity of an infectious process in acute myeloid leukemia patients with neutropenia. Ter Arkh 2013; 85(11): 34-40.
19. Togbe D, Aurore G, Noulin N, Quesniaux VF, Schnyder-Candrian S, Schnyder B, et al. Nonredundant roles of TIRAP and MyD88 in airway response to endotoxin, independent of TRIF, IL-1 and IL-18 pathways. Lab Invest 2006; 86(11): 1126-1135.
20. Rogers GB, Zain NM, Bruce KD, Burr LD, Chen AC, Rivett DW, et al. A novel microbiota stratification system predicts future exacerbations in bronchiectasis. Ann Am Thorac Soc 2014; 11(4): 496-503.

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