Original article:

Impact of promoter CD14 C>T 159 gene single nucleotide polymorphism and outcome of sepsis.

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Abstract:-

Introduction: A genetic polymorphism has been identified inside the CD14 Promoter sequence. It consists of a C to T transition at base pair -159 from the major transcription site. Subjects carrying the T allele have been shown to have significantly higher soluble CD14 levels than do carriers of the C allele. Consequently, genetic variations in CD14 particularly polymorphism located on the promoter region are thought to have functional effects and increased susceptibility to sepsis. ***Methods:*** Our study was a case control study in which a total of 85 samples were included out of which 50 were sepsis free controls and 35 cases of sepsis. Both the cases and controls were selected from Surgical Intensive Care Unit of Sher-i-Kashmir Institute of Medical Sciences (SKIMS), Srinagar. Age more than 80 years, cardiac failure, liver insufficiency and cancer patients were excluded from the study. 5ml of blood from peripheral vein was obtained from each subject in EDTA containing vials and DNA extraction was done by salting out method. ***Results:*** The TT genotype frequency was significantly higher in sepsis than in control (P=0.025) and appeared to be genetic risk factor for increased susceptibility to sepsis. The frequency of mutant T allele observed in cases was 36(51.4%) and 35(35%) in controls. This observation showed a highly statistically significant of rare allele T between cases and controls (P=0.033). Patients with increased Total Leucocytes Count (TLC) were more significantly associated with combined (CT+TT) against CC against those patients with normal TLC (P<0.01). The cases had a higher frequency of the rare allele (CT+TT = 80%) than the controls (64%) and this difference showed statistically insignificant association with CT + TT combination against CC (P=0.11). ***Conclusion:*** The present study suggested that CD14-159C>T may be a risk factor for the development of sepsis in Kashmiri Population.

Keywords: Sepsis; CD 14; Single Nucleotide Polymorphism (SNP); Outcome.

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Introduction:

Sepsis causes millions of deaths globally each year[1]. In the USA sepsis affects approximately 3 in 1000 people a year[2]. It is the second leading cause of death in non-coronary intensive care units and the tenth most common cause of death overall[3]. Sepsis is common and serious in the elderly, immuno-compromised and critically ill patients. It occurs in 1–2% of all hospitalized patients and accounts for as much as 25% of Intensive Care Unit bed utilization.

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Although the patho-physiology of this syndrome is complex, an important breakthrough in understanding the molecular mechanisms of septic shock has been the discovery of CD14 as a receptor for a very wide range of microbial products including lipopolysaccharide, peptidoglycans, and lipoteichoic acid[4]. CD14 is a glycosyl phosphatidyl inositol membrane-anchored protein mainly expressed on the surface of macrophages and monocytes and also on polymorphonuclear leukocytes. It acts in concert with the recently discovered mammalian Toll-like receptors to discriminate between microbial pathogens or their products and initiate transmembrane signal[5,6]. The soluble form of CD14 also plays a crucial role in the microbial response of endothelial and epithelial cells that do not exhibit membrane bound CD14[7]. Thus CD14 is a major part of innate immune system. Several groups have reported an increase in serum CD14 concentrations in human gram-negative and gram positive septic shock patients with an associated increased mortality rate[8,9].

A genetic polymorphism has been identified inside the CD14 promoter sequence[10,11]. It consists of a C to T transition at base pair-159 from the major transcription start site. Subjects carrying the T allele have been shown to have significantly higher soluble CD14 levels than do carriers of the C allele[12,13]. Consequently, genetic variations in CD14, particularly polymorphisms located on the promoter region, are thought to have functional effect. In the present study, a population based case-control study was conducted to examine the association between the CD14 promoter C-159T Single Nucleotide Polymorphism and sepsis in Kashmiri population.

Methods:-

The study was conducted at Sher-i-Kashmir Institute of Medical Sciences (SKIMS). Age more than 80 years, Cardiac failure (Class III and IV), Liver insufficiency (Child C), Bone marrow aplasia, Immuno suppression (Positive human immunodeficiency virus, current immunosuppressive therapy including corticosteroids) and Cancer patients were excluded from the study.The study included 85 blood samples among which 50 were controls & 35 were cases of sepsis. A written pre informed consent was obtained from all cases and controls. 5 ml of blood from peripheral vein was obtained from each subject in EDTA containing vials and was stored at -20^0 C and DNA extraction was done by salting out method.Variables measured in all patients were white blood count, platelet count, coagulogram, blood culture, kidney function test, heart rate, temperature, fasting blood sugar. DNA isolated from blood samples by phenol/ chloroform or ammonium acetate extraction method. The concentration of the DNA obtained was measured in a spectrophotometer at 260nm wavelength [Qauntitation]. The quality of the DNA obtained from the tissue specimens and blood samples was analyzed on 1% agarose gel. CD-14 target gene encompassing 159 C > T SNP was amplified by polymerase chain reaction .Using Specific enzyme AVaII, SNP polymorphism was identified by gel electrophoresis.

The study was a case control study, we compared all the categorical variables with the help of appropriate statistical tests like Chi Square test and Fisher exact test. All the statistical results were discussed as 5% level of significance that is p value less than 0.05 considered significant. In otherwords, we are 95% confident about the results.

Ethical approval: this study was approved by the ethics committee of Sher-i-Kashmir Institute of Medical Sciences (SKIMS)

Observations:

The sex distribution among cases (n-=35) included 25 males (71.42%) and 10 females (28.57%) while as in control group (n=50) there were 32 males (64%) and 18 females (36%). The sex distribution in case and control groups were compared and found to be statistically insignificant (p=0.473) [Table 1]. In both the groups when various variables temperature , heart rate [Table 2] total leucocyte count , platelet count , liver function tests, kidney function tests, blood cultures, fasting blood Sugar where measured were statistically significant. This comparison was also statistically significant. [Table 3]

Table 1: Demographic variables in cases and controls

		Cases (n=35)	Cases (%)	Controls (n=50)	Controls (%)	P value
Age (Yrs)	≤45	15	42.85 %	22	44%	**0.917**
	>45	20	57.14%	28	56%	
Sex	Male	25	71.42%	32	64%	**0.473**
	Female	10	28.57%	18	36%	
Dwelling	Rural	16	45.71%	28	56%	**0.35**
	Urban	19	54.28%	22	44%	

Table 2: General Variables in cases and controls

		Cases (n=35)	Cases (%)	Control (n=50)	Controls (%)	P value
Temp	≤100 ^{0}F	18	51.43%	49	98%	**<0.001**
	>100 ^{0}F	17	48.57%	01	2%	
Heart Rate (bpm)	≤90	11	31.42%	38	76%	**<0.001**
	>90	24	68.57%	12	24%	

Table 3: Laboratory parameters in cases and control

		Cases (n=35)	Cases (n %)	Control (n=50)	Controls (n %)	P value
TLC	Normal	5	14.28%	50		**<0.001**
	Increased	25	71.42%	00	100%	
	Decreased	5	14.28%	00		
Platelet	≤1 lac	23	22.85%	18	36%	**0.007**
	>1 lac	12	34.28%	32	64%	
Coagulogram	Normal	10	28.57%	49	98%	**<0.001**
	Deranged	25	71.42%	01	2%	
LFT	Normal	13	37.14%	50	100%	**<0.001**
	Deranged	22	62.85%	00		
KFT	Normal	18	51.42%	50	100%	**<0.001**
	Deranged	17	48.57%	00		
Blood Culture	Culture Sterile	7	20%	50	100%	**<0.001**
	Culture Positive	28	80%	00		
Blood Sugar (F)	≤90	1	2.85%	18	36%	**<0.001**
	>90	34	97.1%	32	64%	

High molecular weight DNA isolated from the blood samples (cases and controls) Figure 4.1. These were subjected to Polymerase chain reaction to amplify the CD 14 gene -159 C>T in the promoter region. The representative picture of the amplified products is given in the Figure 4.2 .The amplified product was subsequently subjected to digestion with respective enzyme as described in the methodology section and the representative picture of the digested products is given in the Figure 4.3a and Figure 4.3b.

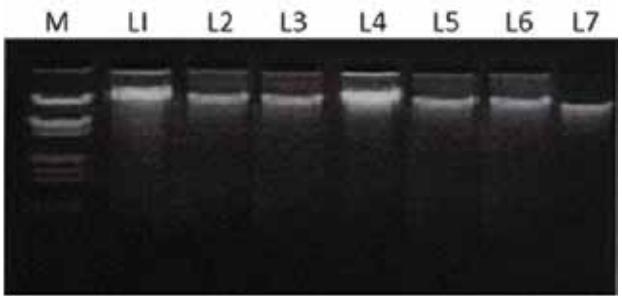

Figure 4.1: 1% Agarose gel electrophoresis of DNA isolated from blood of cases and controls L1-L7.

Lane M consists of lambda DNA *Eco*RI and Hind III digest.

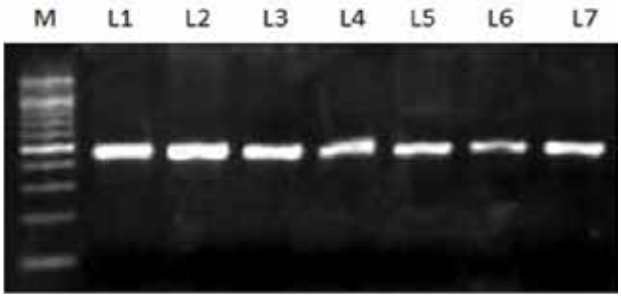

Figure 4.2: PCR amplification of 497 bp CD14 gene encompassing -159 C>T

Lane M: Molecular size marker 100bp

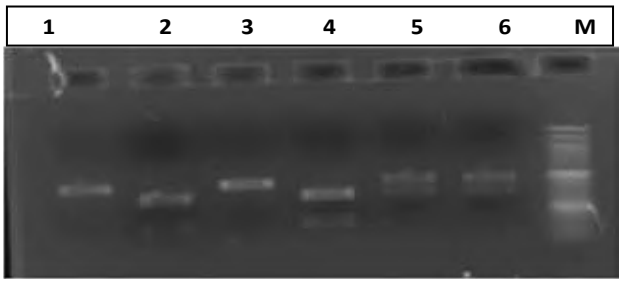

Figure 4.3a : Restriction fragment length polymorphism pattern of CD 14 -159 C>T using AvaII restriction enzyme.

Lane M: 100bp ladder

Lane 1, and 3 represent the Homozygous wild (CC) genotype (size 497 bp)

Lane 2 and 4 represent homozygous variant (TT) genotype) size 353bp+144 bp)

Lane 5 and 6 represent CT heterozygous genotype (size 497, 353 and 144bp)

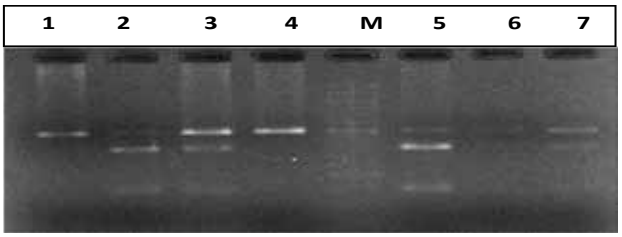

Figure 4.3b : Restriction fragment length polymorphism pattern of CD 14 -159 C>T using AvaII restriction enzyme.

Lane M: 100bp ladder

Lane 1, and 4 represent the Homozygous wild (CC) genotype (size 497 bp)

Lane 2, 3, 5 and 7 represent CT heterozygous genotype (size 497, 353 and 144bp

For CD14-159 C>T Single nucleotide polymorphism a total of 35 sepsis cases and 50 sepsis free controls were studied for polymorphic analysis. The distribution of CD14-159 C>T genotypes and its allele frequency in cases and controls are shown in Table 4. Owing to the very low frequency of the 'TT' genotype and an increased risk associated with CT and TT genotypes, CT + TT was compared against CC. The cases had a higher frequency of the rare allele (CT + TT= 80%) than the controls (64%) and this difference showed statistically insignificant association with CT + TT combination against CC (P=0.11) [Table 4 and chart 11]. CD14-159 C>T Single Nucleotide Polymorphism, frequencies of CC, CT and TT genotypes among controls were 18(36%), 29(58%) and 03(06%) while in sepsis cases allele frequencies were 07(20%), 20(57.1%) and 08(22.9%) respectively with Odds Ratio =6.85 (95% Confidential Interval = 1.40-33.570)Table 5 Bar Chart 1. A higher frequency of mutant T allele observed in cases was 51.4% and 35% in controls Table 5 Bar Chart 2. This observation showed a highly statistical significance of rare allele (T) between cases and controls (p=0.033).

When classified further into groups, our study interestingly found higher number of rare allele (CT + TT) in patients belonging to rural areas (P=0.038) [Table 6]. Patients with increased total leukocyte count (TLC) were more significantly associated with combined (CT +TT) against CC in comparison to those patients with decreased total leukocyte count with Odds Ratio=11.6(95% CI= 1.27-95.178) (p=0.04). It was seen that sepsis patients were more significantly associated with combined (CT +TT) against CC in patients having deranged coagulogram odds ratio=11(p=0.01). Also a significant association with Blood culture was seen with combined (CT +TT) against CC with Odds Ratio=11.11(p=0.01)

Table 4: Genotypic frequency of CD -159 C>T in various characteristics of sepsis cases and controls

	Controls	CC n (%)	CT+TT n (%)	Cases	CC n (%)	CT+TT n (%)	OR (95%CI)	P value
Overall genotype	50	18(36%)	32(64%)	35	07(20%)	28(80%)	2.3 (1.0-4.1)	0.11
Sex								
Female	18	10	8	10	02	08	5.0 (3.0-8.1)	0.11
Male	32	08	24	25	05	20	1.6(0.8-4.1)	0.65
Age group								
<45	22	09	13	15	04	12	2.5(1.0-4.6)	0.28
≥45	28	09	19	20	03	16	2.8(1.2-5.1)	0.35
Dwelling								
Rural	28	12	16	16	04	14	10.2(8.0-64.1)	**0.038**
Urban	22	06	16	19	03	14	1.2(0.3-4.0)	1.0

Table: 5 Genotypic and allelic frequencies of CD 14 -159 C>T in cases and controls

SNP	Cases n= 35	Controls n=50	Odds Ratio (95% CI)	P-Value
CD14 C>T				
Genotype				
TT	08(22.9%)	03(06%)	(ref.)	
CT	20(57.1%)	29(58%)	3.86 (0.912 -16.386.)	0.055
CC	07(20%)	18(36%)	6.857 (1.40 -33.570)	**0.025**
Allele type				
T	36(51.4%)	35(35%)	(ref.)	
C	34(48.6%)	65(65%)	1.966(1.054 -3.668)	**0.033**

Table 6: Genotypic frequency of CD -159 C>T in various characteristics of sepsis

	Cases n=35	CC n (%)	CC+CT n (%)	Odds Ratio (95%CI)	*P* value
TLC					
Normal	05	02	02	11.6(1.27 -95.178)	**0.04**
Increased	25	04	22	6.0(0.35 -101.56)	0.2
Decreased	05	01	04		
Coagulogram					
Normal	10	05	05	11.0(9.0 -17.1)	**0.01**
Deranged	25	02	23		
Blood culture					
Sterile	07	04	03	11.11(1.634 -75.564)	**0.01**
Positive	28	03	25		
LFT					
Normal	13	05	08	6.2(0.99 -39.09)	0.07
High	22	02	20		
KFT					
Normal	18	04	14	1.33(0.251 -7.084)	1.0
High	17	03	14		

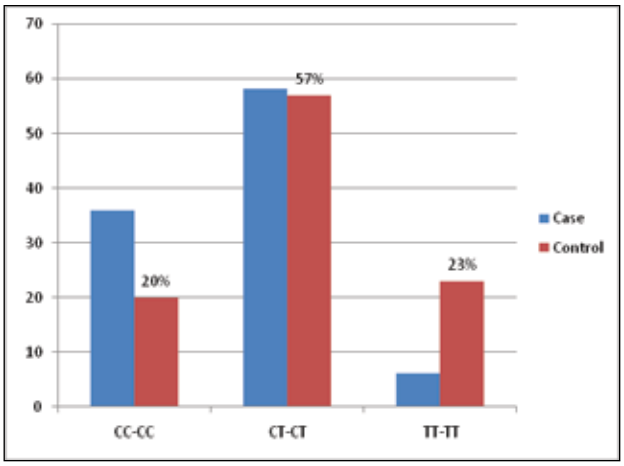

Chart 1 : Distribution of CC, CT and TT genotypes among cases and controls

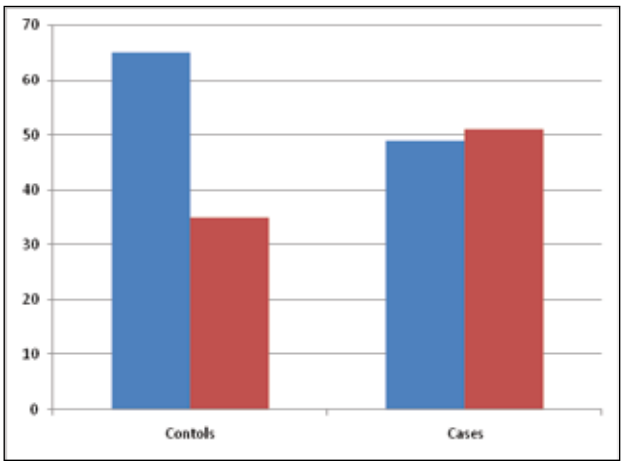

Chart 2: Allele frequency among cases and control

Discussion:

Sepsis remains a major global health problem with a high mortality despite major advances in the care of critically ill children and adults. Genetic variability within the genes responsible for the initial recognition step and the subsequent immune response have been implicated in the variability seen in response to infection and could potentially influence the overall susceptibility to and outcome from sepsis. Though there is no doubt that most polymorphisms are functionally neutral, some affect regulation of gene expression or the function of the coded protein [(14)] .

With this background we conducted a study to evaluate the frequency of this recently discovered CD 14 promoter gene polymorphism (C to T transition at base pair- 159) among patients with sepsis and compared them with those in sepsis free control group. A total of 85 samples included in the study (50 controls and 35 confirmed cases of sepsis) were selected.

Demographic variables and clinic-pathological characteristics of each patients were recorded. The demographic variables including age, sex and dwelling did not show any statistical significance when compared between the two groups. In both the groups temperature, heart rate, total leucocyte count, platelet count, fasting blood sugar, coagulogram, and blood cultures when compared showed high statistical significance.

The distribution analysis of three genotypes in our study revealed genotypic frequency of CC, CT and TT among controls as 18(36%), 29(58%) and 03(06%), while in sepsis cases frequencies were observed as 07(20%), 20(57.1%) and 08 (22.9%) respectively. This study clearly demonstrated a significant association of CD -159 C>T genotype with the susceptibility of sepsis. The TT genotype frequency was significantly higher in the studied sepsis group than in the control population and appeared to be a genetic risk factor for increased susceptibility to sepsis with Odds Ratio (OR) =6.8, Confidence Interval (1.40-33.570) (p = 0.025). The frequency of T allele observed in cases was observed to be 51.4% as compared to 35% in controls and this difference was highly significant (P=0.033). Our report noticed combined CT and TT genotypes were frequently presented in culture positive cases as compared to sterile ones with statistically high significance (p=0.01).

Furthermore, in this study cases with higher Total Leucocyte Count (TLC) had a higher combined CT and TT genotypes than the patients with normal or decreased Total Leucocyte Count and the distribution of variant genotypes among cases with increased TLC showed significance (P=0.04) over those with normal Total Leucocyte Count cases. The result of our study was in accordance with another study conducted by Sebaston Gibot et al and Barber RC et al[15,16].

Moore KJ et al[17] also found CD14 to be an important part of the innate immune system, initiating antimicrobial response. The result of our study was in accordance with other studies conducted by Mauro Baldini et al[18], Paulo R. V. Fallavena et al[19] & Richard L et al[20].

Identification of genes implicated in the susceptibility to common polygenetic and multifactorial diseases is a new and very exciting topic of medical research. Reports of the association of polymorphisms with diseases, such as this study, have increased in number rapidly during the last years by Mc Guire W et al[21], Goldfred AE et al [22] , Iacovillo L et al[23], Lander ES et al[24].

In our study patients and controls were age- and gender- matched. Moreover, the frequency of the T allele in the control group (35%) was close to the reported frequencies of the previously studied by French Sebastin et al[16], German Unkelbach K et al[9], Czech Hubacek JA et al[11], and American Baldini M et al[10] healthy groups. Furthermore, in neither group did the genotype frequency distribution differ from that expected in a population in Hardy-Weinberg equilibrium. An elevation of soluble CD14 concentration is strongly associated with an increased risk of death during septic shock[5,8] . Soluble CD14 promotes microbial components binding to endothelial and epithelial cells[7] .The ensuing activation of these cells may be detrimental and thus may explain the worse prognosis in patients with increased soluble CD14. This relationship suggests that serum concentrations of soluble CD14 may be crucial for the fate of endothelial and epithelial cells.[25]

The present study suggested that CD 14 -159 C>T has been seen a risk factor for the development of sepsis in a Kashmiri population. However, these correlations need to be authenticated in a large sample study in the future due to less number of studies, so as to help in the better discernment of racial differences and in determining the course of sepsis.

Summary:

Genetic variations in CD14, particularly polymorphisms located on the promoter region, are thought to have functional effects and modulate the risk with TT genotype for sepsis and this was the hypothesis on the basis of which we designed this case control study and had the following outcome summarized below.

1. A total of 35 sepsis cases and 50 sepsis free controls were studied for polymorphic analysis of CD14-159 C>T Single Nucleotide Polymorphism.
2. With respect to age, sex and dwelling the sepsis patients and controls were comparable. The difference observed between the two groups was statistically non- significant.
3. In both the groups temperature, heart rate, total leucocyte count, platelet count, fasting blood sugar, coagulogram, and blood cultures when compared showed high statistical significance.
4. CD14-159 C>T Single Nucleotide Polymorphism, frequencies of CC, CT and TT genotypes among controls were 18(36%), 29(58%) and 03(06%) while in sepsis cases allele frequencies were 07(20%), 20(57.1%) and 08(22.9%) respectively with Odds Ratio (OR) =6.857(95%CI=1.40-33.57).
5. The TT genotype frequency was significantly higher in cases than in control which appeared to be a genetic risk factor for increased susceptibility to sepsis with odds ratio 6.857 (95% Confidence Interval = 1.40-33.570) (P=0.025).
6. The cases had a higher frequency of the rare allele (CT + TT= 80%) than the controls (64%) and this difference showed statistically insignificant association with CT + TT combination against CC (P=0.11).
7. The frequency of mutant T allele observed in cases was 36(51.4%) and 35(35%) in controls. This observation showed a highly statistical significance of rare allele (T) between cases and controls (p=0.033).
8. Patients with increased total leukocyte count were more significantly associated with combined (CT + TT) against *CC* against those patients with normal Total Leucocyte count (p<0.01).
9. It was seen that sepsis patients were more significantly associated with combined (CT +TT) against CC in patients having deranged coagulogram odds ratio=11(p=0.01).
10. Also a significant association with Blood culture was seen with combined (CT +TT) against CC, Odds Ratio=11.11(p=0.01)

Conclusion:

The present study suggests that CD 14 -159 C>T may be a risk factor for the development of sepsis in a Kashmiri population. However, these correlations need to be authenticated in a large sample study in the future due to less number of studies, so as to help in the better discernment of racial differences and in determining the course of sepsis. Furthermore, delineation of genetic polymorphisms of CD14 together with Tumor Necrosis Factor-alpha is associated with outcome in sepsis may be useful as a prognostic tool. It could be possible that a rapid bedside assessment of immune response genotype would allow a patient-specific target therapy, improving the unacceptably high mortality rate in sepsis Clinically, our data suggest that the CD14 C159T polymorphism could have a significant role in the evolution and final outcome of septic shock. Further studies including larger population and critically ill controls are needed to confirm the relevance of the C-159T polymorphism.

Conflict of interest: None declared

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