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ORIGINAL RESEARCH ARTICLE

Role of Asp299Gly single nucleotide polymorphism of TLR4 gene in susceptibility of idiopathic dilated cardiomyopathy

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ABSTRACT

Introduction: cardiovascular diseases remain one of the major reasons of universal mortality and accounts for 31% of deaths alone in India. Cardiomyopathy is one such cardiovascular anarchy that is linked with heterogenous aetiologies leading to ventricular hypertrophy and myocardial dysfunction. Between 2008-2015, owing to cardiomyopathies there has been significant increase in evidences of hospitalisation for heart failure, mechanical circulatory supports, defibrillators implantations and 51% of all heart transplants [Lannou Set al, 2020].

Toll-like receptor-4 (TLR4) is mapped in 9q32-q33 is a family member of pattern recognition receptors. Activation of TLR4/ NF-κB induces the expression of ROS stimulating the expression of inflammatory factors [Asehnoune K et al, 2004] causing the progression of cardiac hypertrophy and injury by Cardiomyocyte inflammation followed by apoptosis and fibrosis [Katare PB et al, 2020].

It has been speculated that TLR4 mutations alter the function of infiltered immune cells. It collectively alters myocyte contractility and extracellular matrix regulation leading to altered LV function and dimension during the progression of cardiomyopathy (Alexander Riad et al., 2012). Single nucleotide polymorphism resulting in variants of TLR4 with replacement of conserved aspartic acid residue with glycine at amino acid 299 alters the extra cellular domain of the receptor (El-Zayat et al., 2019).

Materials and method: This study has been carried out In Department of Biochemistry MKCG medical college, Berhampur, Odisha, India. We have examined the association of Asp299Gly SNP of TLR4 gene with dilated cardiomyopathy. Total 63 idiopathic dilated cardiomyopathy patients along with age and sex matched 70 healthy individuals as controls has been included in this study. Genomic DNA was isolated using non-enzymatic method. The gene polymorphism was analysed by PCR followed by RFLP method taking NCoI restriction enzyme.

Results:

The disease prevalence is similar in both the sexes in our study.

For TLR4 Asp299Gly gene polymorphism, the AA genotype was found to be higher in controls than DCM cases (61.43% vs 44.44%, p = 0.014). Cases with GG genotype and combination of AG + GG genotype has a significantly higher risk of getting DCM as compared to controls [OR=0.195 (95% CI= 0.049-0.773) P=0.028 and OR=1.98 (95% CI= 0.996-3.976) P=0.026 respectively]. G allele shows 3.17 times more risk of developing DCM (p= 0.001) than normal population.

Conclusion: Homozygous GG genotype and Heterozygous AG of TLR4 299A>G have been associated with risk DCM and its complications. Hence the current study suggests that TLR4 polymorphism may be a useful diagnostic biomarker in determining the prognosis and risk stratification of the disease among patients as well as aid in screening among the first-degree relatives of patients.

Key words: Dilated Cardiomyopathy, Toll like receptor4, Single nucleotide Polymorphism.

INTRODUCTION:

Cardiovascular diseases remain one of the major reasons of universal mortality and accounts for 31% of deaths alone in India. Cardiomyopathies are relevant cause of heart failure leading to increased morbidity and mortality (Schultheiss HP et al., 2019). It is a cardiovascular anarchy that exhibits improper ventricular dilatation and contractile dysfunction due to an array of causes. Dilated cardiomyopathy being a commonest cause of cardiomyopathy is third most common cause of heart failure (Towbin JA et al., 2006).

The prevalence of heart failure caused due to dilated cardiomyopathy is estimated to be approximately 1: 250-400 and up to 1: 2500 in the general population (Weintraub R.G et al.,2017). In India there were 106,460 new cases of DCM in 1990. In one and a half decades, incidence increased massively and 150,507 new cases were reported in the year 2005. A spike of 207,268 new cases were reported in the year 2019.

When myocardial injury occurs due to any of the causes, some myocytes damages as a result of apoptosis. Dynamic remodeling of remaining myocytes and interstitial scaffold leads to ventricular dilatation and progression to dilated cardiomyopathy. Congestive cardiac failure, usually left ventricular systolic failure is the most common clinical presentation.

The phenotype of ventricular dilatation and abnormal contractile function can be caused due to pathological conditions like ischemia, infection, toxins, metabolic causes or autoimmune disease, however in 50% of cases the aetiology remains unknown and is termed as idiopathic dilated cardiomyopathy. Certain hereditary conditions and altered innate immune response may contribute to its pathophysiology (Feldman A.L and McNamara D, 2000). Activation of innate immune system resulting in cytokine activation is considered as one of pathophysiological mechanism of DCM (Abraham J.Pet al., 2021). The prevalence

of genetic aetiology of cardiomyopathies continue to rise with increasing availability of genetic testing.

Toll like receptors are a family of immune proteins located in large number of cell types. Human TLR4 was the first mammalian Toll protein to be characterized. The human TLR4 gene maps to chromosome 9q32–33, and is composed of four exons and three introns. It is secreted from the ER. Then it is trafficked to cis-Golgi in a coat protein II complex coated vesicles in association with its chaperone molecules, coreceptors and adaptor protein. Then it is exported to plasma membrane where it responds to ligands and trigger a series of inflammatory cascade (Gay N J et al., 2014).

The TLR4/NF-κB signaling pathway is activated by signals such as lipopolysaccharide (LPS) and reactive oxygen species (ROS). Its downstream transcription factor nuclear factor kappa-B (NF-κB) plays an important role in the occurrence and development of inflammation, exerting a variety of effects on heart structure and function (Mann D.L, 2011, Jiedong Zhou et al.,2022).

It has been speculated that TLR4 mutations alter the function of infiltered immune cells which performed trans endothelial migration into cardiac myocytes. It collectively alters myocyte contractility and extracellular matrix regulation leading to altered LV function and dimension during the progression of cardiomyopathy (Alexander Riad et al., 2012). Also, cardiac specific immune effect of TLR4 mutations suggests difference in function of myocytes, cardiac endothelial cells and fibroblast cells (Alexander R et al., 2011).

The host inflammation is initiated by pattern recognition receptors (PRRs), that are essential components of the innate immune system. TLRs are a family of pattern recognition receptors that serve as the first line of defence against invading microbes and/or tissue injury (Curtiss et al., 2009). Ten TLRs have been identified in humans, among which TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10 are located on the plasma membrane, and TLR3, TLR7, TLR8, and TLR9 are expressed in the cytoplasm. The signal transduction of TLRs occurs mainly through myeloid differentiation factor 88 (MyD88) and Toll/Interleukin-1 receptor domain-

containing adaptor-inducing interferon-β (TRIF). Some differences are observed among TLR subtypes: all TLRs except TLR3 require MyD88 to be recruited to the TIR domain. TLR3 and TLR4 ligands recruit the linker protein TRIF, whereas TLR2 and TLR4 signals require not only MyD88 but also Mal/TIRAP cooperation (Mifsud EJ et al., 2014, Brennan JJ et al., 2018).

TLR4 located in the cell specifically recognizes bacterial lipopolysaccharide, along with several other components of pathogens and endogenous molecules produced during abnormal situations, such as tissue damage. It is known to play a key role in recognizing pathogen associated molecular pattern and in initiating cytokine activation in cardiac cells and other cell types. Andit has been asserted that distorted expression of innate immunity genes causes an imbalance in pro and anti-inflammatory cytokines that is involved with unremitted course of cardiac dysfunction (Edfeldt K et al., 2002). TLR4 activates the expression of several proinflammatory genes that play pivotal roles in myocardial inflammation, particularly myocarditis, myocardial infarction and heart failure. Cardiac TLR4 activation by pathogenic lipopolysaccharides increases the Ca2+ efflux via the sodium- calcium exchanger and prolongation of action potential duration. This lands up in arrythmogenic events in heart failure (Milberg P et al., 2012, Monnerat-Cahil G et al., 2014). In this gene, SNP rs4986790, also known as 896A/G, is a non-synonymous mutation characterized by substitution within the 3rd exon of the TLR4 gene, from an adenine (A) to a guanine (G) at position 896 (896A>G) which leads to modification of the conserved residue of aspartic acid to a glycine in amino acid 299 of the protein sequence (Asp299Gly) on, in or at the domain of the extracellular structure of TLR4.

Studies have shown that functional polymorphism in TLR4 gene has contributed to acute coronary syndrome signifying its effect in modulating inflammatory responses, the strength of which is genetically determined. This study has tried to elucidate the effect of SNP on TLR4 gene (299 A>G) towards heart failure caused due to dilated cardiomyopathy.

MATERIALS AND METHODS:

This was a case control study and carried out in patients diagnosed with idiopathic dilated cardiomyopathy, attending to the Department of Cardiology and Medicine, M.K.C.G Medical College, Berhampur, Odisha. This study has been approved by IEC.

Inclusion criteria: The cases were composed of adults > 18 years of age, diagnosed as idiopathic dilated cardiomyopathy (DCM) by cardiologists, including both males and females. Patients with increased left ventricular end-diastolic diameter (LVEDD >58 mm) and reduced ejection fraction (LV EF) from 2D echocardiography were considered as cases of dilated cardiomyopathy.

Exclusion criteria: Patients with other disorders like Hypertension, Coronary artery disease, type 2 DM, Thyroid dysfunction, long standing arrythmia, valvular heart diseases, Acute infection and sepsis, Chronic renal disease, Congenital heart diseases, Pulmonary artery disease, Acute viral myocarditis, Connective tissue disorder and autoimmune diseases were excluded from our study.

Age and sex matched randomly selected healthy subjects without any history of cardiac or systemic disorder were included as controls. Clinical examination including measurement of systolic blood pressure (SBP) and diastolic blood pressure (DBP) was applied. Anthropometric measurements (weight and height) were collected and used for BMI calculation according to the standard formula BMI=weight (kg)/[height(m)]2.

Five ml of venous blood was collected in a sterile syringe and was taken in a EDTA vacutainer for isolation of DNA after taking written informed consent from the study participants.

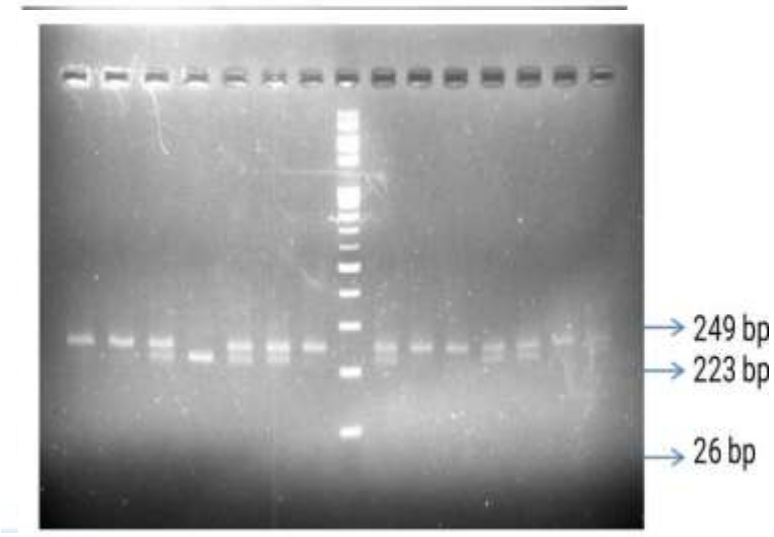
Laboratory analysis:

Genomic DNA was isolated from whole blood by using phenol chloroform method. Quality of isolated DNA was checked in Nano-drop (Denovix). Along with the quantity check, the isolated DNA has also been checked and confirmed by 0.8% agarose gel electrophoresis followed by gel documentation. Gene polymorphism study was carried out for TLR4 Asp299Gly by PCR followed by RFLP

method using NCoI restriction enzyme. All the reaction was carried out as per the previously published research article (Bagheri et al., 2015). The visualization of amplified PCR product by 3% agarose gel electrophoresis was carried out and compared with 100 bp ladder. A 249 bp amplified product was visualized where amplification has been performed. Then NCoI restriction digestion was done.

The analysis of RFLP product is as follows. Gel doc Picture attached

- 249bp: Presence of both 'A' allele only (AA genotype)
- 249bp+223bp+26bp: Presence of both 'A' and 'G' alleles (AG genotype)
- 223bp+26bp: Presence of 'G' allele only (GG genotype)



3%agarosegelelectrophoresisfortheNCoI digestedgene(Asp299Gly)polymorphism

Statistical Analysis:

All the data were presented by number, percentage, mean and standard deviation. Hardy-Weinberg equilibrium for the allelic frequency was calculated by using Chi square test of significance. The comparison of distribution of genotypes among the groups was analysed by using Pearson's Chi square test of significance. A P value of < 0.05 is considered to be statistically significant.

RESULTS:

There were 63 cases and 70 healthy controls recruited in this study. We found that there were no significant differences in occurrence of the disease among males and females.

Table 1.0 showing Gender wise distribution of cases with DCM and control.

Gender	Cases with DCM (n=63) Number, %	Control (n=70) Number, %	Pearson χ2	P Value
Male	40 (63.49)	49 (70.0)	χ2=0.634	P=0.426
Female	23 (36.51)	21 (30.0)		

P< 0.05 is considered significant

Table 2.0 showing Comparison of mean age between cases with DCM and control

Age	Cases with CM (n=63) Mean ± SD	Control (n=70) Mean ± SD	T test	P Value
Age (In years)	54.44±10.5	53.39±12.49	t=0.526	P=0.60

P< 0.05 is considered significant

The mean age of the cases with DCM was 54.44±10.5 years and the mean age of the control group was 53.39±12.49 years. There were no differences in the mean age of the cases with DCM and control. (P=0.60).

Table 3.0 showing Distribution of TLR4 Asp299Gly genotype and allele frequency in DCM cases and controls.

TLR4 Asp299Gly genotype	Cases with DCM (n=63) Number, %	Control (n=70) Number, %	Odds ratio OR [95% CI]	P value
AA	28 (44.44)	43 (61.43)	0.49 (95% CI 0.285-0.846)	**0.014**
AG	25 (39.68)	24 (34.29)	0.625 [0.299-1.304]	0.285
GG	10 (15.87)	3 (4.29)	0.195 [0.049-0.773]	**0.028**
AG + GG	35(55.55	27(38.57)	1.98[0.996-3.976]	**0.026**
G allele	33(52.38)	18(25.21)	3.17 [1.53-6.58]	**0.001**

P< 0.05 is considered significant; CI- confidence interval

For TLR4 Asp299Gly gene polymorphism, the AA genotype was found to be higher in controls than DCM cases (61.43 vs 44.44%, p = 0.014). Cases with AG genotype has a higher risk for the disease [OR= 0.625, 95% CI 0.299-1.304, P=0.285]. Cases with GG genotype and combination of AG + GG genotype has a significantly higher risk of getting DCM as compared to controls [OR=0.195 (95% CI 0.049-0.773) P=0.028 and OR=1.98 (95% CI 0.996-3.976) P=0.026 respectively]. G allele shows 3.17 times more risk of developing DCM (p= 0.001)

DISCUSSION:

This study revealed that the genotype and allele frequencies for TLR4 gene polymorphism (rs4986790) between cases with DCM and controls were in

agreement with those predicted by the Hardy-Weinberg equilibrium for all the TLR4 gene polymorphism in cases and controls.

Studies have shown that the Toll like receptors, such as TLR4 are basically genes that encodes transmembrane receptors responsible for inducing a pro or anti-inflammatory response. Human beings with TLR4 polymorphisms are observed to be hyporesponsive to several ligands (**Michelsen KS et al., 2004**). Our study has shown that the allelic frequency of 'A' in cases is 0.31 and in controls it is 0.69. This has able to suggest that Controls with 'A' allele has a significantly reduced risk of DCM in comparison to 'G' allele [OR= 0.49 (95% CI 0.285-0.846) P= 0.014].

The homozygous ancestral allele AA was used as a reference to determine the possible risk of acquiring Dilated cardiomyopathy was associated with other two mutant genotypes AG and GG. Our TLR 4 findings in our control subjects are within the variation seen in various populations for this rare SNP (**Sarli A et al., 2017**).

According to **Semlali et al. (2019**), the distribution of genotype frequencies for rs 4986790 between patients with cardiovascular diseases and healthy controls was similar, that was more appreciable in GG and AG genotypes (frequency of AG genotype in cases and control of 14% and 11% respectively). Our study found similar result for AG genotype (frequency of AG genotype in cases and control of 39% and 34% respectively).

The role of TLR4 single nucleotide polymorphisms in increased risk of dilated cardiomyopathy have been studied (**Bardiaa A et al., 2016**). Harnesneimi, et al, 2008 reported that 299Gly allele are linked with extensively high carotid artery elasticity in a cohort of 2201 young adults.

TLR4 single nucleotide polymorphism causes a missense that results in disturbance in protein folding is being studied by Bardiaa A et al., 2016. This may induce differences in pathogenicity of dilated cardiomyopathy. In humans, polymorphisms of TLR 4 influences the extracellular domain of the molecule and these individuals are hyporesponsive to numerous ligands due to altered cytokine responses. Anti infllammatory response of TLR4 is masked and pro inflammatory

action predominates. Systemic inflammatory response to low-grade infectious stimuli leads to progression of cardioyopathy by two mechanisms

- TLR4 Asp299Gly polymorphisms blunt the inflammatory response
- leading to ineffective removal of infectious agents, and persistence/ progression of the low inflammatory trigger.

Moreover, neointimal formation is diminished in TLR4 Asp299Gly polymorphisms cases, causing neointimal lesion. So, an increased risk of Cardiomyopathy is seen in individuals with a hypo-responsive TLR4 genotypes (AG and GG).

Conclusion:

Homozygous GG genotype and Heterozygous AG of TL4 299A>G have been associated with risk of DCM and its complications. Hence the current study suggests that TLR4 polymorphism may be a useful diagnostic and prognostic biomarker in DCM subjects. This SNP may also helpful in risk stratification among patients as well as aid in screening among the first-degree relatives of patients.

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