Mutation Screening of MEFV and TNF Gene in Pakistani Patients with Rheumatic Heart Disease: A Case Control-Study

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Abstract

Background: Rheumatic heart disease (RHD) is an inflammatory autoimmune cardiovascular disorder. The disease is highly prevalent in both urban (22 per 1000 individuals) and rural (5.7 per 1000 individuals) areas of Pakistan. The main purpose of this research work was to examine the role of two most widely studied genes, MEFV and TNF in the susceptibility of RHD in Pakistani patients.

Methods and Materials: In total 360 samples, including 156 clinically diagnosed RHD patients and 204 healthy controls were included in the study. Single strand conformational polymorphism (SSCP) and direct DNA sequencing approach were used to identify the genetic changes in TNF exons and hot spots of MEFV gene.

Results: No genetic variation in the two genes was detected in this study except a novel mutation (g.G2,096A) in exon 2 of MEFV gene. Computational analysis revealed that this mutation (p.S179N) severely affect the three-dimensional structure of the protein and thus probably has a pathogenic role. However, this mutation was identified in two patients only.

Conclusion: Hence, contribution of this mutation is expected to be very small in Pakistani patients. Our results showed a novel mutation with pathogenic effect in a very small proportion of the RHD patients in Pakistan. However, majority of the patients may have mutation outside the hot spot region of MEFV gene or there are other susceptibility factors that are contributing toward high prevalence of RHD in Pakistan. Therefore, it is important to screen the complete MEFV gene and other genetic susceptibility factors to understand etiology of RHD and thus manage its increasing incidence.

Keywords: Rheumatic heart disease; MEFV; TNF alpha; Autoimmune; Pakistan

Introduction

Mediterranean fever gene (MEFV) and tumor necrosis factor (TNF) are crucial for inflammatory processes. MEFV (protein product, Pyrin) plays important role in regulating inflammatory process, hematopoiesis, oncogenesis and embryonic development [1]. The role of pyrin in inflammation is to direct the movement of white blood cells to the site of inflammation in order to stop or slow inflammatory process. It communicates with other molecules involved in fighting against infection and regulating inflammatory response [2]. Further, involvement of MEFV in FMF suggests that this gene may also have a role in susceptibility of RHD [3]. Based on this hypothesis, MEFV gene has been screened in RHD patients in few populations based studies with varying results [4,5]. These observations indicate that there could be population specific susceptibility factors for RHD.

Tumor necrosis factor (TNF) is an important multifunctional pro-inflammatory cytokine. It is involved in the regulation of different biological functions such as inflammation, cell proliferation, differentiation, apoptosis, lipid metabolism and coagulation [6]. TNF gene has shown high level of variability within itself and is located in highly polymorphic region, human leukocyte antigen (HLA). The production of this pro-inflammatory cytokine in response to infection is partly controlled during transcription; for which genetic polymorphisms play an important role. Such as, raised level of TNF is associated with promoter polymorphism (G-308A) in RF patients [7,8]. Therefore, polymorphisms in TNF gene have also been explored in different studies as RHD susceptibility factor [9-11]. Despite the fact that Pakistan has the 4th highest prevalence of RHD in the World, scare data about the genetic susceptibility of RHD is available (WHO, 2011). Previously, we studied different polymorphisms in the IL10, IL1RN, TNF and HLA [12,13]. Here, we explore the whole exons of TNF and hot spot regions of MEFV gene.
Materials and Methods

In total, 360 individuals including 156 RHD patients and 204 normal healthy individuals were recruited for the study. RHD patients 127 females and 29 males; mean age 31 ± 14.10 years were collected from three different health institutes; Shifa International Hospital (SIH), Islamabad, Pakistan Institute of Medical Sciences (PIMS), Islamabad and Armed Forces Institute of Cardiology (AFIC), Rawalpindi. All the RHD patients were with mitral valve disease, had history of RF and were in quiescent or chronic phase. They fulfilled the two dimensional M-mode and Doppler echocardiography criteria for mitral valve disease [14].

The control group comprised of 204 healthy individuals 108 females and 96 males, mean age 45 ± 12.7 years with normal echocardiogram and no history of RF. The patients and the controls were matched ethnically. They were from various castes and tribes of Northern Punjab and Khyber Pakhtunkhwa (KPK) in Pakistan. Venous blood samples were collected after informed written consent of the individuals or their legal guardians. The study protocol was approved by respective institutional bioethics committees of IBGE and SIH. DNA was extracted from blood using a standard organic extraction protocol [15].

In order to screen the samples for hot spot regions in exon 2 and exon 10 of MEFV and for all the four exons of TNF gene primers were designed using web-based program Primer3 (Table 1 supplementary material). PCR was performed using standard protocol and 100 ng of genomic DNA was used in final volume of 25 μl. All the primer sets had the same first step of 94°C for 3 min, followed by 35 cycles of 94°C for 45 s, extension at 72°C for 45 s and 72°C for 10 min, while the annealing temperature and concentration of MgCl₂ varied from primer to primer. The PCR products were denatured at 95°C and run on 12% acrylamide gel to detect any conformational change that reflects change in the nucleotide within amplified genomic region [16]. Samples were carefully examined for mobility shift of bands and Sanger sequencing was done wherever required to confirm the sequence change.

In an attempt to predict the impact of this mutation on the structure and thus function of MEFV, we predicted the 3-D structure of the wild-type and mutant protein using I-Tasser [17]. STITCH4 database [18] was used to predict functional protein partners. Docking analysis was carried out using Patch Dock server [19,20]. The refinement of first 10 docked complexes obtained through PatchDock was done using FireDock [21]. Representations (2-Dimensional) and analysis of protein-ligand interaction complexes was done using LIGPLOT [22].

Results

Two hot spots; exon 2 and 10 of MEFV gene and for all the four exons of TNF-a gene were screened for polymorphism in RHD samples. Samples were carefully examined for mobility shift of bands and sequencing was done where required but no polymorphism associated with Pakistani RHD patients was observed for these two genes. However a novel nucleotide change was detected in exon 2 of MEFV gene at position g.G2,096A (Figure 1). Two female RHD patients were found to carry this mutation in homozygous state; however, the mutation was not detected in any of the control sample. This nucleotide change alters the codon and results in change of amino acid (Serine to Asparagine; p.S179N). The mutation lies between the Pyrin (PYD) and BBox domain (Figure 1c). No other mutations were detected in any of the other study participants.

Model prediction and docking analysis confirmed that mutation altered the folding and conformation of MEFV protein. Figure 2 shows 3-dimensional models for wild and mutant proteins. Protein ligand PSTPIP1 (Proline-Serine-Threonine Phosphatase Interacting Protein 1) with maximum interaction score (0.995) with MEFV was selected for docking analysis in reference to results obtained through Stitch4 database. It is involved in regulation of the actin cytoskeleton. It may physically couple CD2 and CD2AP to W AS. Wild and mutant types of MEFV were docked with PTPN12 to study the docking interaction and mutation effect. Figure 2 shows three dimensional visualization of docking complexes using viewer ite. Ligplot results for the docking interactions of wild and mutant type MEFV with ligand PTPN12 are shown in Figure 3 part A and B, respectively. Residues involved in hydrogen bonding and hydrophobic interactions during docking are given in Table 1. Mutation although altered the single nucleotide but it caused huge impact on structure and conformation of protein as
compared to normal protein. This can be well observed through differences in number and position of residues involved in both types of docking interactions. This is due to alteration of active site after mutation which effected protein's interaction with ligand and in turn functions of protein.

| Receptor-Ligand  | Hydrogen Bond Interactions | Hydrophobic Interactions |
|------------------|-----------------------------|--------------------------|
| **Wild MEFV - PTPN12** | Gln198, Arg737 | Leu194, Glu195, Gly196, Gly197, Ala736, |
|                  | Asp23, Ser68            | Phe54, Ser67, Gly69      |
| **Mutated MEFV - PTPN12** | Gly4, Ser5, Gly7, Ala29, Gly30, Ile36, Leu37, Glu35, Glu40, Asp41, Thr43, Thr45, Phe54, Lys62, Leu63, Ser64, Gly65, Pro66, Ser67, Ser68, Gly69, Leu10, Ser38, Pro41, Arg42, Met53, Thr101, Asp102, Ala105, Ala106, Ser108, Pro115, Thr326, Leu396, Ile397, Thr62, Leu770, Ile772 |
|                  | Asp103, Asn113, Asp170   | Leu10, Ser38, Pro41, Arg42, Met53, Thr101, Asp102, Ala105, Ala106, Ser108, Pro115, Thr326, Leu396, Ile397, Thr62, Leu770, Ile772 |

Table 1: Receptor (MEFV) and Ligand (PTPN12) residues involved in interactions.

**Figure 2:** Visualization of docking complex. **A)** MEFV with PTPN12 **B)** Mutant MEFV with PTPN12. Receptor shown with Ca wire display style and pink color, ligand: schematic display style.

**Figure 3:** 2D Ligplot representation of Docking Interaction. **A)** Normal MEFV with PTPN12, **B)** Mutant MEFV with PTPN12.

**Discussion**

*MEFV* and *TNF* gene product play a key role in immune system and there malfunction through any change in their gene results in the abnormal immune response. *MEFV* gene product pyrin is involved in the regulation of inflammation, variants of *MEFV* gene alters its
function and results in certain inflammatory and autoimmune disorders [23,24]. In a study on 27 Turks RHD patients MEFV mutations were screened and the result showed that the MEFV mutations gene frequency was four times greater in RHD patients than in the normal controls [4,5].

Our result differs from this study as we found no mutation or polymorphism associated with RHD in Pakistani population. There could be several reasons for this discrepancy, one of these is that our group is considerably large n=156 as compared to their 27 patients, secondly the RHD samples they used were relatively of young age ranging from 7 and 18, while our patients mean age range was from 31 ± 14.10 years. Another reason might be that they used sequencing technique and we used SSCP technique. Differences in geographical background can also be another reason. The mutations which were studied in Turks occurred in FMF patients, it might be suggested here that our study gives evidence that no association exists between FMF and RF. A novel nucleotide change was detected in exon 2 of MEFV gene at position g.G2096A which is positioned between the Pyrin (PYD) and BBox domain in our study. The PYD is involved in inflammation and apoptosis [25]. It interacts with other proteins like NLR (Nod-like receptor) and ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain) to form a large multiprotein complex known as inflammasome [26]. The inflammasomes activates inflammatory pathways and is crucial for host defense against pathogens. Mutations in the PYD domain can influence the interaction of pyrin with ASC and alters the activation of inflammasomes thus leading to auto-inflammatory disorders. Although the mutation detected in this study (p.S179N) is not present in PYD domain; however, it greatly altered the overall 3-D structure of the protein and may potentially influence the formation of inflammasomes. Docking analysis showed interaction site in mutated protein changes which effected protein's interaction with ligand and in turn function of protein. The commonly studied MEFV mutation p.E148Q shows some changes in the PRY and SPRY domain (Figure 1, Supplementary material). These minor structural effects in p.E148Q mutant protein are in concordance with the previous findings and therefore exhibit inconclusive phenotypic effect [27,28]. Considering the impact of p.S179N novel mutation on 3-D structure of MEFV, it is expected that this mutation may have stronger phenotypic effect compared to the reported mutation (p.E148Q) in the proximity. The p.S179N mutation was detected in two patients only and the rest of the patients did not show any mutation in the 2nd or 10th exon of the MEFV gene.

TNF plays an important role in initiation, maintenance and regulation of immune system. Its role in various disease etiologies has been studied and is well documented [29].The level of this important cytokine varies according to the disease conditions. Several promoter polymorphisms are detected in it, which are said to be associated with susceptibility to different diseases including RHD. [13,29]. Due to diverse action and important location of the TNF-α, we designed the primers for its 4 exons to see whether any polymorphism in its gene is associated with etiology of RHD among Pakistani Patients but we were unable to find any polymorphism within this region associated with the disease, to our knowledge this was the first time when the TNF-α exons are screened in RHD patients for genetic variations.

Limitations

Our study has some limitations including small sample size and use of SSCP which is not advanced molecular biology technique like Sequencing.

Conclusion

Our results show that p.S179N novel mutation has pathogenic effect in a very small proportion of the RHD patients in Pakistan. However, majority of the patients may have mutation outside the hot spot region of MEFV gene or there are other susceptibility factors that are contributing toward high prevalence of RHD in Pakistan.

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Conflict of Interest

All the authors declare that they have no conflict of interest regarding the publication of this manuscript.

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