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ASSESSMENT OF SINGLE NUCLEOTIDE POLYMORPHISIM rs1888747 IN *FRMD3* and rs6930576 IN *SASHI1* GENES ON DIABETIC NEPHROPATHY PATIENTS IN PAKISTANI POPULATION

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Diabetic nephropathy (DN) is the major complication of type I and II diabetes. This condition then eventually leads to end stage renal disease (ESRD). It is commonly observed that there is close association between the single nucleotide polymorphism (SNPs) and DN. Studies have also evaluated the significant association of SNPs at *FRMD3* and *SASH1* locus with diabetic kidney disease (DKD). To find out the same association in Pakistani population, we conducted a study with the aim to characterize the genomic polymorphism in *FRMD3* and *SASH1*gene. For this purpose, 30 blood samples were collected from the Mayo hospital Lahore, Pakistan, of which 20 samples were included study group (of DN patients) and 10 samples were of control group (of healthy individuals). After sample collection, DNA was extracted through organic method. Gel electrophoresis (2%) was done for quantitative and qualitative analysis. Samples amplified through PCR were then sequenced and phylogenetic tree was constructed to perform gene comparison in different organisms. The results of our study showed that SNP rs1888747 in *FRMD3* is associated with DN but SNP rs6930576 in *SASH1* is not associated with diabetic nephropathy in Pakistan. These two risk allele expression did not differ considerably in case and control groups respectively. It is suggested that the genetic predisposition of Pakistani population of diabetes differs considerably to Japanese and

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European population. Therefore, further studies need to be conducted with more number of patients in Pakistan to find out the stronger association between these SNPs and Diabetic nephropathy.

Keywords: Diabetic Nephropathy, SNP r1888747, SNP rs6930576, *FRMD3, SASH1*

INTRODUCTION

Diabetic nephropathy is microvascular problem of diabetes (SZOSTAK *et al*., 2023) which is categorized by obistinate albuminuria and inexorable deterioration in glomerular filtration rate (GFR) (SULTAN *et al*., 2023). Conventionally, DN has five recognized stages which depend on urinary albumin secretion and glomerular filtration rate (GRF): glomerular hyperfiltration, a silent stage, incipient nephropathy with microalbuminuria, overt nepheropathy and, which leads to end stage renal disease (ESRD). Diabetic nephropathy is multifactorial disorder caused by Diabetes mellitus type 1 and type 2. It is linked with sickness and mortality (RIZVI *et al*., 2014). According to the NDSP (National diabetic survey of Pakistan) report 27.4 million cases reported in Pakistan (DIMEGLIO *et al*., 2018). DN causes renal distraction at glomerular capillaries and tubular interstitium SAGOO and GNUDI (2018). It raises the chances of cardiovascular disease (CVD) as compared to non-diabetic common people (DOMANSKI *et al*., 2002; HU *et al*., 2022). In diabetic nephropathy kidney blood capillaries damage due to high glucose level and high blood pressure. Protein come out form the blood capillaries to the urine due to which end stage renal failure occur that leads to diabetes nephropathy (SANDHOLM *et al*., 2012). DN has multifactorial pathogenesis and complex trait as similar to the complications of DM. Genetic susceptibility of DN has appeared as an important aspect for pathogenesis. Genetic susceptibility to DN indicate several lines of evidence (MOOYAART, 2014). The SNP which is accompanying with the development of Diabetic nephropathy is located near the promoter region. This scenario provides a remarkable suggestion for the transcriptional regulation and genomic variation in the context of diabetic nephropathy (KELLER *et al*., 2012).

FRMD3 gene is positioned on chromosome 9, band q21.30. SNP r1888747 is located near the promoter region of *FRMD3* gene. The strongest association of SNPs with DKD was detected at *FRMD3* (4.1 protein ezrin, radixin, moesin, firm containing domain 3) locus. *FRMD3* gene encodes structural protein 4.10 which is the part of protein 4.1 family (CHANG *et al*., 2015; BUFFON *et al*., 2016). FERM domain containing 3 (FRMD3) helps in maintenance of the shape and integrity of nephron cells, and bone morphogenetic protein 7 (BMP7) helps maintain function and reduce kidney damage (MOHAMMADI *et al*., 2023). *FRMD3* SNP (rs1888747) associated with DKD which include in severe nephropathy development.*FRMD3* gene is the powerful candidate for DN. Molecular identification of *FRMD3* gene may increase understanding about pathogenesis (AL-WAHEEB *et al.*, 2016). Diabetic ESRD patients had inferior existence than non-diabetic ESRD patients. The study described that hereditary disposition is important risk factors for DN development (LIAO *et al.*, 2014).

SASH1 gene is positioned on chromosome 6. SASH1 features a 25-residue proline-rich region at the N-terminal. This gene's mRNA has a three-prime untranslated region with two polyadenylation signals. SASH1 gene shares 85% homology in human and mouse (JANSSEN, 2014). SASH1 belongs to the SLY family, and its closest homologues are SLy1 and SLy2 (SH3-domain containing protein expressed in lymphocytes) (LIM, 2014). SASH1 codes for a protein

that can be found in both the cytosol and the nucleus (GURLEY, 2018). The SASH1 gene has two transcripts of roughly 4.4 and 7.5 kb, which are mostly transcribed in the human breast, thyroid, lung, spleen, placenta, and thymus (NAZAR, 2014). SASH1, and other related molecules regulates cytoskeletal proteins and promotes adhesion of the cell and matrix. In addition it was found that SASH1 affected E-cadherin signaling for the regulation of the trans-epithelial migration (VAN et al., 2000).

The SNP rs6930576, which is found in the gene's intron, has the strongest link to T2DM ESRD (TZIASTOUDI *et al*., 2017). The SNP rs1888747 of *FRMD3* gene has been associated with DN in many studies conducted worldwide but there is no schematic study conducted against *FRMD*3 in Pakistan. Therefore it was necessary to conduct a study that may help in disease diagnosis and treatment.

MATERIALS AND METHODS

Sample collection

The study was started by approval of ethical committee (University of Veterinary and Animal Sciences, Lahore) and informed consent of the patients after thorough explanation of purpose of the study. In addition to that all the participants were assured that their personal information will kept confidential. There was no relationship between Diabetic Nephropathy patients with other group. Control group samples were collected from healthy individuals. A total (n=30) human blood samples was collected from Mayo hospital Lahore, Pakistan. Out of those, 20 blood samples were taken from Diabetic Nephropathy Patients (study group), 10 blood samples were collected from healthy person (control group). Control group include individuals with no history of Diabetic neuropathy, renal or hepatic diseases. Sampling was done between January to March 2021. Samples were collected in an EDTA covered vacutainer. After sample collection, the blood samples were placed in ice and then transferred to the Molecular lab and was stored at -20°C prior to DNA extraction.

DNA isolation and primer designing

DNA was extracted by using organic method which is also known as phenol chloroform extraction. In this process first step was lysis of cells so lysis buffer was used and blood samples were centrifuged at 13000 rpm for 15 min. This step was repeated three to four times to achieve a whitish pallet and after it was incubated overnight with 10% SDS, proteinase K and buffer A1. Next day equal amount of PCI (Phenol: Chloroform: Isoamylalcohol) was added and centrifuged at 15000 rpm for 8 min, supernatant was discarded and chilled isopropanol was added. DNA was precipitated and DNA was again centrifuged (at 13000 rpm for 15 min) and supernatant was discarded. The last step was air drying of samples.

Quantitative and Qualitative Analysis of DNA: Nano drop spectrophotometer was used to check 260/280 ratio for protein contamination and 260/230 ratio for chemical contamination. 1µl of DNA sample was used for analysis while 1µl of distilled water was used as blank solution. Genomic DNA was visualized by using 2% agarose gel. DNA sample of 3µL volume was mixed with 2µL of 5X bromophenolblue, a loading dye. After mixing, it was loaded into the wells. Gel was run for 35 minutes at 111V. After that agarose gel was seen under UV light in Bio-Rad gel documentation system.

Primer designing and Conventional PCR: Primers (forward and reverse) were designed using Primer3 software for the amplification of SNP 1888747 of *FRMD3* gene and rs6930576 in *SASH1* gene (Table 1). FASTA format of both the genes was retrieved from NCBI. Then sequences with some flanking region from start to end were taken and copied and pasted on primer 3 software. Specificity of primers was checked at OligoCalcs software including melting temperature, hairpin, heterodimers, self-complementarity and GC content. Primers were optimized with gradient PCR by changing temperature and concentrations. PCR was performed for temperature optimization ranging from 53-63°C. After optimization of the primer by gradient PCR, samples were processed using conventional PCR. Following conditions were set in conventional PCR i.e. denaturation at 94°C for 30sec, annealing at 63°C for 30sec and extension at 72°C for 45sec for 35 cycles. The volume of each reaction mixture was 25µl. Gel electrophoresis was performed and samples were run on 2% agarose gel for 35min to find out the successful amplification of DNA sequences. When visualized under UV light by gel documentation system (Bio Rad), DNA bands were seen. As per bands on the gel amplification at 63°C was better than others as that band was sharpest and without any smears (SHARIF *et al*., 2022).

Table 1. Sequences of forward and reverse primers designed by Primer3 plus.

Name	Sequence	Melting temperature	Product size
r1888747 of *FRMD3* gene			
Forward Primer	ACAGGCTCTGGAAGCACTT	57.5°C	197
Reverse Primer	TTTTCTTTCAGTGGTCCAGTTTT	56.5°C	197
rs6930576 in *SASH1* gene			
Forward Primer	CGGAAAGAAGCGCAGAAAT	55°C	250
Reverse Primer	CCGTCTTTCATACAACCAAC	56°C	250

Sequencing of PCR products: Amplified PCR products were sent for DNA sequencing using Sanger's sequencing through commercial facility, a local company ABI (Genetic analyzer 3130). Results of DNA sequencing were then analyzed on Bioinformatics software Chromas (version 2.6.6). All the unreadable and mislabeled peaks were deleted with help of CodonCode analyzer. FASTA format of the sequenced region were with the reference sequence of the DNA in BLAST NCBI. https://blast.ncbi.nlm.nih.gov › Blast. Sequences were analyzed from electropherogram to find out SNPs in the region. Phylogenetic trees of both genes were then constructed by using Clustal W software to find out the presence of similar genes in various organisms.

Statistical analysis

Statistical analysis was done by using the SPSS (Statistical Package for Social Sciences) version 22.0 for windows 8. In statistical analysis we had done the chi square analysis to check the association of SNP, hardy Weinberg equilibrium for sake of comparison and measure that whether the observed genotypic frequencies differ from the expected genotypic frequencies to determine statistically significant differences between the groups. Chi-Square test was run for this variable and the value of chi-square came out to be .073 and .567 and p value (asymptomatic

significance 2-sided) obtained was 1 i.e. p-value is >0.05 and hence, it is considered statistically non-significant.

RESULTS

Current study was aimed to characterize the genomic polymorphism in *FRMD3* and *SASH1* gene as marker for association of Diabetic Nephropathy. PCR product was analyzed on 2% agarose gel-electrophoresis. Product size obtained for *FRMD3* gene was 197bb when compared with ladder. Similarly, *SASH1* gene was obtained from product size of 250bp, as shown in the (Figure 1).

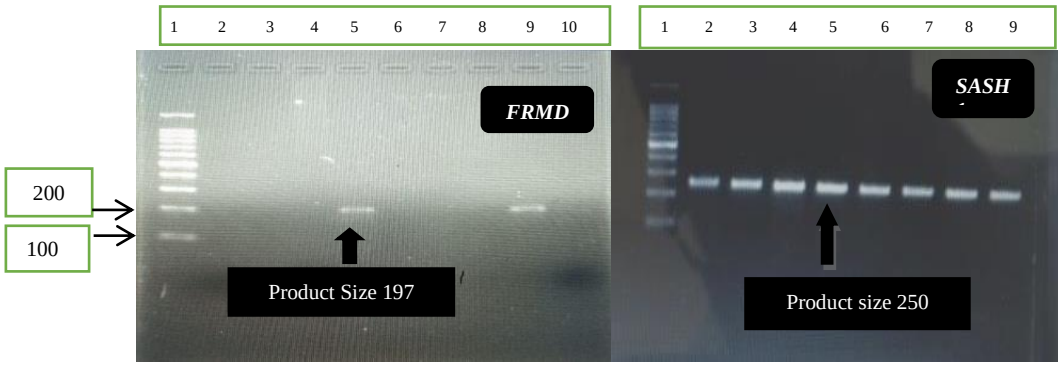

Figure 1. PCR Product of *FRMD3* and *SASH1* gene visualized through gel documentation system (The numbring above the figure shows the number of well)

Table 2. Genotypic distribution and P-values of SNPs in FRMD3 and SASH1 genes

Location	Gene	SNPs	Position	Genotype	Genotypic mutations	Allele Frequency of population	P-value
Chromosome 9	*FRMD3*	rs1888747	83540636	AG	AC	G - 0.175 C - 0.825	.073
Chromosome 6	*SASH1*	rs6930576	148383818	CG	CA	G - 0.605 A – 0.395	.567

In case of *FRMD3* gene (P-value .073), it was observed the genotypic mutation in nucleotide from AG to AC. The allelic frequency of "G" nucleotide in population was 0.175; similarly the value of same parameters for "C" nucleotide was 0.825. Similarly, genotypic mutation for *SASH1* gene was CA from CG with population frequency of G (0.605) and A (0.395). It is clearly observed from the results that allele C show the highest value of allele frequency in the population (Table 1).

Retrieval sequence of FRMD3 gene

After sequencing of DNA nucleotides for both the genes, DNA sequences were retrieved by using the Chromas software. Following is the retrieval sequences of *FRMD3* gene of diabetic nephropathy patients.

AGCTGGTATTACCAAGTATGGGTCACCTGGGATTGAACTACCCAATGAAGACAAGG
CTCTGGGAAACCAACTGGCCATTGTCAACAATAATAATAATAATATGAAGATTAGG
AAGAACTCAAAGAATGAACTCAAGGGATGAGTTTAAAACTGGACCACTGAAAGAA
AAA

Chromatogram of Diabetic Nephropathy Patient

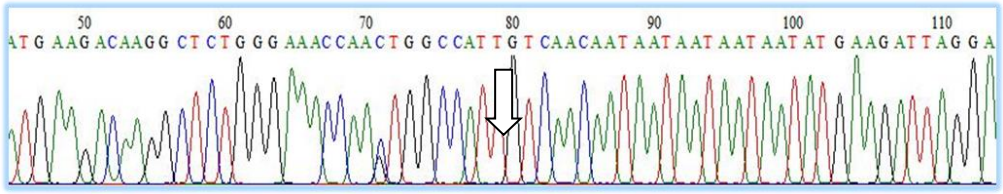

Figure 2. Chromatogram of DN Patients obtained from Chromas

The chromatogram is clearly showing the heterozygosity at reported SNP named as rs1888747 at the location of 83540636. The arrow is indicating the heterozygous peak AC which is genotypic mutation in the patients of diabetic nephropathy having *FRMD3* gene. The normal patients have the nucleotide sequence AG at this location of the gene (Figure 2).

Phylogenetic Tree

Sequence alignment was done by using CLUSTAL W. Phylogenetic trees were constructed to find out the presence of both genes in various organisms and their association.

a) *FRMD3*gene

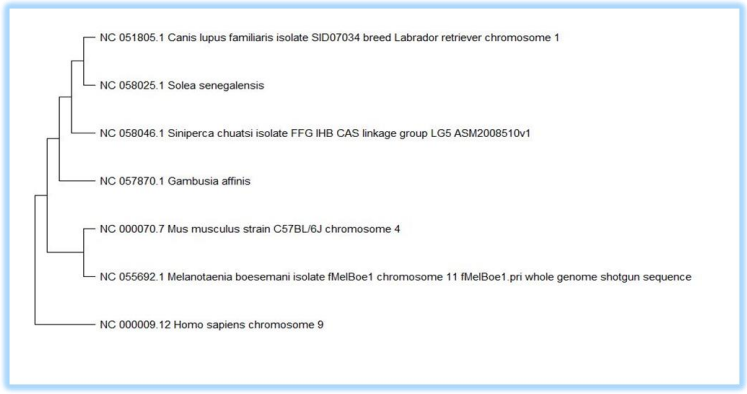

Figure 3. Phylogenetic tree of *FRMD3*gene showing the homology between the different organisms

FRMD3 gene is present in the several type of species like in human its cause Diabetic Nephropathy. Figure 3 shows the presence of gene *FRMD3* gene in following species like *Mus musculus* (house mouse) at chromosome 4, *Melanotaenia boesemani* (boesemanrain bow fish) located at chromosome 11, *Gambusia affinis* (western mosquito fish) chromosome LG03, *Siniperca chuatsi* (mandarin fish) located at Chromosome LG5, *Solea senegalensis* (Senegalese

sole) Chromosome at LG5 and Canis lupus familiaris (dog). We should need to know either that gene cause diabetic nephropathy in these organisms or not. In human, *FRMD3* SNP (1888747) shows the strong association with diabetic nephropathy.

b) *SASH1* gene

Figure 4. Phylogenetic tree of *SASH1* gene showing the homology between the different organisms

SASH1 gene in Homo sapiens is not associated with the diabetic nephropathy in population of Pakistan but that gene is present in several species. In Figure 4 show the following are the species in which this gene is present, i.e. in Pan troglodytes (chimpanzee) this gene is located at chromosome 6, in Xenopus tropicalis (tropical clawed frog) it is located at chromosome 5, in Rattus norvegicus (Norway rat) it ispresent at chromosome 1, while Macaca mulatta (Rhesus monkey) it is present at chromosome 4, in Macaca mulatta (Rhesus monkey) present at chromosome 9 and in Mus musculus (house mouse) it is present at chromosome 10.

DISCUSSION

Diabetic nephropathy showed alteration in kidney mainly in form of renal hypertrophy which eventually leads to glomerular sclerosis and tubulointestinal fibrosis (UD DIN *et al*., 2022). The main pathways that lead to the progression of DN are the formation of reactive oxygen species, activation of protein kinase pathway, connective tissue growth factor and transforming growth factor-β1. Since the pathogenesis of diabetic nephropathy is complex and not fully understood due to which the therapeutic outcomes are very poor (SAMSU, 2021). The risk allele rs1888747 involve in the generation of transcription factor binding site. This site involve in the bone morphogenetic protein pathway (BMP) that leads to progression of DN (PEZZOLESI *et al*., 2013). The association of rs6930576 in *SASH1* gene has been studied in many studies however the structural changes occur in this gene during the pathogenesis of DN need further studies (MCDONOUGH *et al*., 2011; GU, 2019).

Our study evaluated the main genes in which the change in nucleotide leads to diabetic nephropathy. The principle aim of our study was to determine the *FRMD3* and *SASH1* genes association with diabetic nephropathy in Pakistani diabetics. These two risk alleles did not differ considerably between case and control groups; hence our study findings showed association between SNP of *FRMD3* with DN and S*ASH1* genes does not show association with diabetic nephropathy in Pakistani diabetic.

The SNPs in non-protein coding regions of the genome, which may affect regulatory functions (FREEDMAN *et al*., 2011). One such background may be a biological process determined by genes whose transcription is synchronized by common regulatory elements in their promoters (FESSELE *et al*., 2002). The SNP located in one of these regulatory elements may alter or disrupt this coordinated regulation, leading to changes in gene expression and subsequent phenotypes. It is possible to identify this mechanism by altering the transcription factor binding site (TFBS) of candidate SNPs (DEGTYAREVA *et al*., 2021).

PEZZOLESI *et al*. (2009) identified a significant association between SNP rs1888747 in *FRMD3* and DN. But this study was exclusively based on results obtained from type 1 diabetes patients. Here, in 820 cases and 885 control group with type 1 diabetes were included and the investigator genotyped 360,000 SNPs in these patients.

MAEDA *et al.* (2010) reported a strong association between SNPs in *FRMD3* and DN. In this study, type 1 and type 2 diabetes patients were included in order to replicate the previous one.1500 Japanese diabetics with and without diabetic nephropathy. Further, PEZZOLESI *et al*. (2013) collected the sample of 943 patients with diabetic nephropathy (case) and 943 without diabetic nephropathy (control) and studies showed a strong association between SNP rs1888747 in *FRMD3* and DN. Same type of association of *FRMD3* genes with DN is also reported in Pakistan in the region of Burewala and Faisalabad (SHARIF *el al*., 2022).

SASH1 has broad expressions throughout the body.*SASH1* and other related molecules regulate cytoskeletal proteins and promotes adhesion of the cell and matrix. In addition it was found that *SASH1* affected E-cadherin signaling for the regulation of the trans-epithelial migration (VAN OS *et al*., 2000). The SNP rs6930576, which is found in the gene's intron, has the strongest link to T2DM ESRD (TZIASTOUDI *et al*., 2017). Therefore, despite the high expression of risk alleles, SNP rs1888747 in *FRDM3* is associated with DN and SNP rs6930576 in *SASH1* is not associated with diabetic nephropathy in Pakistan. Our study also showed the same results of association of rs1888747 SNP in *FRMD3* gene, in the samples collected in PIMS (Pakistan Institute of Medical Sciences), Pakistan (MAQSOOD *et al*., 2023). Our study evaluated the allelic frequency of allele G with value of 0.175. While, allele G was more abundant than previous one and showed the value of 0.825.

CONCLUSION

The analysis of SNPs with diabetic nephropathy has been described in Arab countries (EZZIDI *et al*., 2009).We are first ones in South Asian countries to report this association between these SNPs rs1888747 in *FRMD3* and rs6930576 *SASH1* genes with DN. In our study, the numbers of patients are very low as compared to studies done previously which could cause the conflicting results obtained. The findings of our study explain that the genetic predisposition of Pakistani diabetes differs a lot to that in Japanese and European diabetes studied previously and reported. Therefore, further studies have to be done with larger number of Pakistani population

to detect significant association between these SNPs and Diabetic Nephropathy. This step could help us to report the genetic predisposition of South Asian to diabetes more precisely. This SNP analysis can be used as a base in the identification of other factors that can be a cause of the DN. The factors responsible for the polymorphism can also be subjected to further analysis of how multiple factors are involved in polymorphism.

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PROCENA POLIMORFIZIMA JEDNOG NUKLEOTIDA rs1888747 U FRMD3 i rs6930576 U SASHI1 GENIMA KOD BOLESNIKA OD DIJABETSKE NEFROPATIJE U PAKISTANSKOJ POPULACIJI

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Izvod

Dijabetička nefropatija (DN) je glavna komplikacija dijabetesa tipa I i II. Ovo stanje na kraju dovodi do poslednje faze bubrežne bolesti (ESRD). Obično se primećuje da postoji bliska povezanost između polimorfizma jednog nukleotida (SNP) i DN. Studije su takođe procenile značajnu povezanost SNP-ova na *FRMD3* i *SASH1* lokusu sa DKD. Da bismo otkrili istu povezanost u pakistanskoj populaciji, sproveli smo studiju sa ciljem da okarakterišemo genomski polimorfizam u *FRMD3* i *SASH1* genu. U tu svrhu prikupljeno je 30 uzoraka krvi iz bolnice Maio Lahore, Pakistan, od čega je 20 uzoraka uključeno u studijsku grupu (od pacijenata sa DN) i 10 uzoraka iz kontrolne grupe (zdravih osoba). Nakon uzimanja uzorka, DNK je ekstrahovana organskom metodom. Za kvantitativnu i kvalitativnu analizu urađena je gel elektroforeza (2%). Uzorci amplifikovani putem PCR-a su zatim sekvencionirani i konstruisano je filogenetsko stablo da se izvrši poređenje gena u različitim organizmima. Rezultati naše studije pokazali su da je SNP rs1888747 u *FRMD3* povezan sa DN, ali SNP rs6930576 u *SASH1* nije povezan sa dijabetičkom nefropatijom u Pakistanu. Ekspresija ova dva alela rizika nije se značajno razlikovala u slučajnoj i kontrolnoj grupi. Pretpostavlja se da se genetska predispozicija pakistanske populacije za dijabetes značajno razlikuje od japanske i evropske populacije. Stoga je potrebno sprovesti dalje studije sa većim brojem pacijenata u Pakistanu kako bi se otkrila jača povezanost između ovih SNP-a i dijabetičke nefropatije.

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