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Association of Heat Shock Protein 70 (HSP-70) and Endothelial Nitric Oxide Synthase (eNOS) in the Pathogenesis of Diabetic Retinopathy in Southeast Asian Population

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Abstract:
Present study was set to investigate the role of genetic polymorphisms of the oxidative and inflammatory pathway genes including Heat Shock Protein 70 (HSP 70) and endothelial Nitric Oxide Synthase (eNOS) in the onset of Diabetic Retinopathy (DR) among the southeast Asian population like Pakistan. A case-control association study was conducted for a total of 553 individuals (among which 160 were DR, 193 DNR while 200 were healthy controls) by screening Single Nucleotide Polymorphisms (SNPs) in HSP70 (promoter region: (-27G>C) through standard Polymerase Chain Reaction (PCR) while the eNOS [exon 7 SNP rs1799983 (c.894G>T; p.Glu298Asp) of eNOS was genotyped through PCR Restriction Fragment Length Polymorphism (PCR-RFLP) and an intron 4 VNTR] promoter regions was screened through standard PCR.For HSP70, a significant association of the C allele was found for diabetic individuals specifically the males (χ^2 =21.30, $p<0.05$, OR=2.58 [95% CI=1.68–3.97, $p<0.05$]) while no association of eNOS (rs1799983 and VNTR) was observed in the studied subject. The current study concluded that HSP70 polymorphism is associated with diabetic retinopathy while, eNOS seemed to have no role in diabetes retinopathy susceptibility in south Asian diabetic patients. Bioinformatic analysis also supported the genetic analysis where lack of association was observed for exonic SNP rs1799983 of eNOS with diabetic retinopathy.

Keywords: Diabetic retinopathy; Heat shop protein; Endothelial nitric oxide synthase; Inflammatory; Oxidative stress

Introduction

Uncontrolled diabetes mellitus leads to vision related complications like retinopathy, cataract and glaucoma while diabetic retinopathy remained top most complication among vision related pathologies [1]. International Diabetes Federation (IDF) have estimated that the number of diabetic patient will increase from 451 million worldwide in 2017 to 691 million in 2045[2]. This increase in diabetes mellitus patients will consequently result in proportional increase in Diabetic Retinopathy (DR) cases. Numerous factors have been identified in the development of DR which include consistent hyperglycemia[3], oxidative stress [4], inflammasome [5], genetic factor [6], renin angiotensin aldosterone system [7], gender [8], sympathetic nervous system [9] and endothelial nitric oxide polymorphism [10]. Hyperglycemia through various upregulations like NADPH oxidase, Nuclear factor erythroid 2-related Factor 2 (NRF2), Advanced Glycation End product (AGE) induced oxidative stress which is major factor in the pathogenesis of diabetic retinopathy [11]. These all upregulated pathways can be blocked by using antioxidant which consequently will arrest the progression of diabetic retinopathy[11]. Oxidative stress is dual edged sword which not only increases free radicals but also induces inflammation by upregulation of inflammasome in the eye which is second factor in the pathogenesis of DR [12]. Early diagnosis of oxidative stress and inflammation in diabetic patients can arrest the progression of DM to DR. NLRP3 inflammasome induces inflammatory mediators (TNFα, interleukin-1β, and inducible nitric oxide synthase, interleukins, NF-kB and AGE), proptosis and microangiopathy and retinal neurodegeneration [13].

Under chronic hyperglycemic condition, reducing sugar reacts with active sites of different protein which forms glycosylation end product which activates NF-KB and receptors of these proteins and increased oxidative stress [14]. During these stressful situation and increased oxidative stress, a conserved proteins, heat shock protein 70 (HSP 70) are expressed in DM [15]. Geranylgeranyl Acetone (GGA) induces HSP 70 levels which has protective role by activating PI3K/AKT/mTOR signaling and reduce Retinal Ganglion Cells (RGCs) apoptosis in a rat retinal I/R model [16,17]. Due to increased leukocytosis, stress in the optic nerve head leading to the activation of Heat Shock Proteins (HSPs) which are molecular chaperones functioning mainly in the prevention of protein aggregation which is very important for the survival of retinal ganglion cells [18]. HSPs have been reported to have diagnostic, prognostic and therapeutic potential in various diseases like irritable bowel disease [19]. Association of HSP70 polymorphism and diabetic nephropathy exist in diabetic patients [20]. Similarly association between HSP 70 and arterial hypertension was also established which reported that anti HSP70 activity in the kidney protect nephropathy [21]. HSP70 is a critical element in the pathogenesis of hypertension by either generating anti-HSP70 immune reactivity and/or driving autoantigens to the MHC in antigen-presenting cells [22, 23]. Initially in many pathological situation like hypertension, HSP 70 fight against the stress but in long term situations HSP 70 induces inflammatory pathway [24]. Heat shock transcription factor have been considered as novel mechanism in the Neurodegenerative Diseases, Inflammatory Bowel Disease, Cancer, male infertility, and fetal alcohol spectrum disorder [25]. However, the exact role of HSP70 in development of diabetic retinopathy patients from southeast Asian population have not been established for diabetes and DR.

Endothelial Nitric Oxide Synthase (eNOS) gene is associated with diabetic macular edema in diabetic patient [26]. Association of eNOS gene polymorphism and diabetic retinopathy in Caucasian is reported [27]. Another study reported that genetic variation at the eNOS locus as genetic risk factor for diabetic retinopathy, which may serve as a useful marker of increased susceptibility to the risk of retinopathy [28]. Polymorphism of eNOS genes is linked with diabetic retinopathy in different races of the world. Data reported that Caucasians with Type 1 diabetes that (i) eNOS4a/a is associated with absent or non-severe DR, and (ii) eNOS4b/b is associated with severe DR [29]. Different genes of endothelial nitric oxide synthase were study in Bahrain which identifies genetic variation at the eNOS locus as genetic risk factor for diabetic retinopathy, which may serve as a useful marker of increased susceptibility to the risk of retinopathy[30]. Different forms of NOS (eNOS, iNOS and nNOS) was blamed in the pathogenesis of DR [31-33]. The intron 4 polymorphism of eNOS is associated with Primary Open Angle Glaucoma (POAG), as well as Primary Closed Angle Glaucoma (PCAG), while the G+190C polymorphism in HSP70 is associated with PCAG, but not with POAG in the Pakistani population [34]. Brain-Derived Neurotrophic Factor (BDNF) and HSP70 F 196G/A and HSP70-1 190G/C gene polymorphisms may be related to progression of POAG in polish population [35].

An association of *eNOS* and *HSP70* polymorphism has been linked with diabetic retinopathy and vision related pathologies in different populations of the world and both genes polymorphism have been linked with different types of glaucoma in Pakistan but limited data is available of its role in pathogenesis of DR and gender predisposition. The current study was therefore focused on determining the role *eNOS* and *HSP70* polymorphisms in the pathogenesis of DM and its induced retinopathy comolication.

Methods

4.1. Subject selection

The present case-control study conformed to the Helsinki declaration and the sample collection was done after an informed written consent. The selected panel (N=553) comprised of persons with diabetic induced retinopathy (DR; N=160), diabetic non-retinopathy (DNR; N=193) and controls (N=200). DR patients were sampled based upon the clinical subtype of the disease i.e. proliferative DR (PDR; N=70) and non-proliferative DR (NPDR; N=90). Both DR and DNR subjects were positive for T2DM with a fasting glucose level ≥106mg/dL and had diabetes for more than ten years. Funduscopy and Fundus Fluorescein Angiography (FFA) ophthalmic tests were used for the diagnosis of DR in diabetic patients and subclinical types in cases i.e. NPDR and PDR.

4.1.1. DNA extraction: The blood samples were stored in Ethylene Diamine Tetra acetic Acid (EDTA) vacutainers and placed at 4^{o}C. The DNA was extracted using phenol/chloroform protocol[36]. Briefly, the protocol consisted of Red Blood Cell (RBC) lysis followed by lysis of White Blood Cell (WBC) and degradation of proteins using SDS and proteinase K. After that, protein extraction was carried out using phenol and Tris-EDTA (TE) leaving behind DNA, which was precipitated using sodium acetate and ice-cold isopropanol. Finally, the extracted DNA was re-suspended in TE buffer and stored at -20^{o}C until further use.

4.1.2. Genotyping: The genotyping of *HSP70* the PCR was performed as previously done [37] while PCR-RFLP was used for genotyping of *eNOS* rs1799983 (F: 5'-AAGGCAGGAGACAGTGGATGGA-3'; R: 5'-CCCAGTCAATCCCTTTGGTGCTCA -3', Tm: 65°C). *eNOS* rs1799983, was genotyped using 4U of *BanII* (*Eco24I*). The *eNOS* VNTR was genotyped through PCR using primers as follows: (Forward primer: 5'-AGGCCCTATGGTAGT -3'; Reverse primer: 5' TCTCTTAGTGCTGTG 3', Tm: 58°C).

4.2. Bioinformatics analysis of *eNOS* exonic SNP rs1799983

In order to determine the effect of exonic SNP rs1799983 of *eNOS* and to predict its pathogenicity in manifestation of DR bioinformatic analysis was done using an online tool HOPE (https://www3.cmbi.umcn.nl/hope/input/).

4.2.1. Statistical analysis: The genotyping data were computed and analyzed using chi square analysis and univariate logistic regression analysis. Results were considered statistically significant with a p value ≤0.05. In order to determine the involvement of selected SNPs in disease development, different comparisons were conducted i.e. DR vs. control and DNR vs. control. In order to determine the role of the SNPs in disease progression, comparisons based on clinical stage of the disease were also performed i.e. PDR vs. control and NPDR vs. control.

Results

In the present case control study, healthy as well as diseased subjects were genotyped for *HSP70* gene polymorphism. In the control group, the frequency of GG, GC and CC genotypes was 56.5%, 27.5% and 16%, respectively. For DR patients, this distribution was 34.7%, 40.6% and 24.6%, respectively, with significant association found for the genotype distribution in the DR patients, as compared to the controls (χ^2=20.07, p<0.05). In DNR patients, the frequency of the GG, GC and CC genotypes were 43.5%, 37.5% and 19%, respectively, with significant association found for the genotype distribution in the DNR patients, as compared to the controls (χ^2=6.97, p<0.05).

In addition to the genotype distribution, the frequency of the G allele was found to be at a significantly lower frequency in both patient groups compared to in the control group, whereas the frequency of the C allele was significantly higher and found to associated with the DR (χ^2 =20.63, p<0.05, OR=1.93 [95% CI=1.44–2.59, p<0.05]) and DNR groups (χ^2=5.72,p<0.05, OR=1.43 [95% CI=1.05–1.94, p<0.05] [Table 1].

Table 1: Genotype and allele frequency distribution of HSP70 gene C\G polymorphism among DR cases, DNR cases and controls.

Genotype	**Control (N=200)**	**DR (N=219)**	**DNR (N=200)**	**Control vs DR**		**Control vs DNR**		**DNR vs DR**	
				χ^2 (p-value)	**OR (95%CI) (p-value)**	**χ^2 (p-value)**	**OR (95%CI) (p-value)**	**χ^2 (p-value)**	**OR (95%CI) (p-value)**
GG	113 (56.5%)	76 (34.7%)	87 (43.5%)	**20.07 (0.00)**	**DM: 2.44(1.62-3.69) (0.00)**	**6.97 (0.03)**	**DM:1.68 (1.11-2.55) (0.01)**	3.86 (0.14)	DM: 1.45 (0.96-2.19) (0.07)
GC	55 (27.5%)	89 (40.6%)	75 (37.5%)		**RM: 1.72 (1.02-2.88) (0.03)**		RM: 1.23 (0.71-2.13) (0.51)		RM: 1.39 (0.85-2.29) (0.19)
CC	32 (16%)	54 (24.6%)	38 (19%)						
Allele frequency									
G	281 (70.3%)	241 (57.6%)	249 (62.3%)	**20.63 (0.00)**	**1.93 (1.44-2.59) (0.00)**	**5.72 (0.02)**	**1.43 (1.05-1.94) (0.02)**	**4.49 (0.03)**	**1.34 (1.01-1.79) (0.04)**
C	119 (29.7%)	197 (44.9%)	151 (37.7%)						
Males	**(N=100)**	**(N=109)**	**(N=98)**	**Control vs DR**		**Control vs DNR**		**DNR vs DR**	
GG	59 (59%)	28 (25.7%)	41 (41.8%)	**23.82 (0.00)**	**DM: 4.16 (2.23-7.82) (0.00)**	**5.84 (0.05)**	**DM: 2.01 (1.09-3.67) (0.02)**	**6.06 (0.05)**	**DM: 2.08 (1.11-3.91) (0.02)**
GC	27 (27%)	54 (49.5%)	38 (38.8%)		RM: 2.02 (0.94-4.39)		RM: 1.47 (0.65-3.36)		RM: 1.36 (0.67-2.80)

CC	14 (14%)	27 (24.8%)	19 (19.4%)		(0.06)		(0.34)		(0.40)
Allele frequency									
G	145 (72.5%)	110 (50.5%)	120 (61.2%)	**21.30 (0.00)**	**2.58 (1.68-3.97) (0.00)**	**5.68 (0.02)**	**1.67 (1.07-2.61) (0.02)**	**4.84 (0.03)**	**1.55 (1.02-2.34) (0.03)**
C	55 (27.5%)	108 (49.5%)	76 (38.8%)						

OR= Odds Ration, DM= Dominant Model, RM= Recessive Model

Table 2: Genotype and allele frequency distribution of HSP70 gene C/G polymorphism among NPDR cases, PDR cases and controls.

Genotype	**Control (N=200)**	**PDR (N=96)**	**NPDR (N=123)**	**Control vs PDR**		**Control vs NPDR**		**NPDR vs PDR**	
				χ^2 (p-value)	**OR (95%CI) (p-value)**	**χ^2 (p-value)**	**OR (95%CI) (p-value)**	**χ^2 (p-value)**	**OR (95%CI) (p-value)**
GG	113 (56.5%)	32 (33.3%)	44 (35.8%)	**14.76 (0.00)**	**DM: 2.59 (1.52-4.46) (0.00)**	**13.38 (0.00)**	**DM: 2.33 (1.43-3.81) (0.00)**	1.91 (0.38)	DM: 1.11 (0.61-2.03) (0.77)
GC	55 (27.5%)	36 (37.5%)	53 (43.1%)		**RM: 2.16 (1.16-4.02) (0.01)**		RM: 1.41 (0.76-2.59) (0.29)		RM: 1.54 (0.79-2.98) (0.21)
CC	32 (16%)	28 (29.2%)	26 (21.1%)						
Allele frequency									
G	281 (70.3%)	100 (52.1%)	141 (51.3%)	**18.66 (0.00)**	**2.17 (1.49-3.15) (0.00)**	**11.24 (0.00)**	**1.76 (1.24-2.48) (0.00)**	1.19 (0.27)	1.23 (0.83-1.84) (0.28)
C	119 (29.7%)	92 (47.9%)	105 (42.7%)						

OR= Odds Ration, DM= Dominant Model, RM= Recessive Model

Moreover, gender-based analysis revealed that the polymorphism is strongly associated in males in DR (χ^2=23.82, p<0.05, OR=4.16 [95% CI=2.23-7.82, p<0.05] as well as in DNR (χ^2=5.84, p<0.05, OR=2.01 [95% CI=1.09-3.67, p<0.05]). But there was no such association found in females [Table 1].

Furthermore, the analysis with PDR and NPDR strongly revealed that the polymorphism is strongly associated with PDR (χ^2=14.76, p<0.05, OR=2.59 [95% CI=1.52-4.46, p<0.05] as well as with NPDR (χ^2=13.38, p<0.05, OR=2.33 [95% CI=1.43-3.81, p<0.05]), indicating that *HSP70* is not only involved in the disease development but also has a role in its further progression [Table 2].

The statistical comparisons of the genotype and allele frequencies of *eNOS* rs1799983 in DR, DNR and control group are shown in Table 3. The genotype distribution in controls was: 61% GG, 36% TG and 3% TT. Whereas 67.5% DR and 66.8% DNR subjects had GG genotype, while 32.5% DR and 33.2% DNR cases had TG genotype. None of the DR or DNR subjects in the studied cohort had the TT genotype. The G allele frequency in control, DR and DNR group were 79%, 83.8% and 83.4%, respectively, while that of the T allele were 21%, 16.3% and 16.6%, respectively. The frequency of the three genotypes among diabetics (i.e. DR+DNR) was: 67.1% GG, 32.9% heterozygous TG while there were no homozygous TT in the studied group. The frequency of the G allele was observed to be 83.6% while that of the T allele was 16.4%. No significant difference in genotype and allele frequencies were observed between the studied group in addition logistic regression analysis did not reveal association of the SNP with the studied groups (Table 3). Similar to the results of *eNOS* rs1799983, no significant association was observed for the *eNOS* VNTR polymorphism in the studied panel (Table 4).

Table 3: The genotype and allele frequencies of *eNOS* rs1799983 in the studied cohorts.

	Controls	DR	DNR	PDR	NPDR	DR vs. Controls	DR vs. DNR	DNR vs. Controls	PDR vs. Controls	PDR vs. DNR	NPDR vs. Controls	NPDR vs. DNR	PDR vs. NPDR
Overall	**N=200**	**N=160**	**N=193**	**N=70**	**N=90**	OR (95% CI)	OR (95% CI)	OR (95% CI)	OR (95% CI)	OR (95% CI)	OR (95% CI)	OR (95% CI)	OR (95% CI)
GG	122 (61%)	108 (67.5%)	129 (66.8%)	47 (67.1%)	61 (67.8%)	DM: 0.75 (0.48 - 1.19) 0.23 RM: 0.09 (0.0-1.30) 0.05	DM: 0.97 (0.61 - 1.55) 0.91 RM: 1.21 (0.0-1271397) 0.45	DM: 0.78 (0.50 - 1.20) 0.25 RM: 0.08 (0.0-1.08) 0.02	DM: 0.77 (0.41 - 1.41) 0.39 RM: 0.21 (0.0-3.01) 0.20	DM: 0.99 (0.53 - 1.83) 1.00 RM: 2.75 (0.0-2906050) 0.27	DM: 0.74 (0.43 - 1.30) 0.29 RM: 0.17 (0.0-2.33) 0.21	DM: 0.96 (0.54 - 1.70) 0.89 RM: 2.12 (0.0-2260261) 0.32	DM: 1.03 (0.50 - 2.11) 1.00 RM: 1.28 (0.0-1363147) 0.44
TG	72 (36%)	52 (32.5%)	64 (33.2%)	23 (32.9%)	29 (32.2%)								
TT	6 (3%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)								
G	316 (79%)	268 (83.8%)	322 (83.4%)	117 (83.6%)	151 (83.9%)	0.73 (0.49 - 1.09) 0.13	0.98 (0.64 - 1.49) 0.92	0.75 (0.51 - 1.09) 0.12	0.74 (0.43 - 1.32) 0.27	0.99 (0.57 - 1.72) 1.00	0.72 (0.44 - 1.18) 0.18	0.97 (0.58 - 1.60) 1.00	1.02 (0.54 - 1.94) 1.00
T	84 (21%)	52 (16.3%)	64 (16.6%)	23 (16.4%)	29 (16.1%)								
Males	**N=100**	**N=77**	**N=94**										
GG	66 (66%)	54 (70.1%)	65 (69.1%)			DM: 0.83 (0.42 - 1.65) 0.63 RM: 0.25 (0.0-5.02) 0.26	DM: 0.96 (0.47 - 1.94) 1.00 RM: 1.22 (0.0-1293696) 0.45	DM: 0.87 (0.45 - 1.65) 0.65 RM: 0.21 (0.0-4.10) 0.25					
TG	32 (32%)	23 (29.9%)	29 (30.9%)										
TT	2 (2%)	0 (0%)	0 (0%)										
G	164 (82%)	131 (85.1%)	159 (84.6%)			0.80 (0.43 - 1.47) 0.48	0.91 (0.48 - 1.73) 0.90	0.83 (0.47 - 1.47) 0.59					
T	36 (18%)	23 (14.9%)	29 (15.4%)										
Females	**N=100**	**N=83**	**N=99**										
GG	56 (56%)	54 (65.1%)	64 (64.6%)			DM: 0.68 (0.36 - 1.30) 0.23 RM: 0.13 (0.0-2.0) 0.06	DM: 0.98 (0.51 - 1.89) 1.00 RM: 1.19 (0.0-1263343)	DM: 0.70 (0.38 - 1.28) 0.25 RM: 0.11 (0.0-1.67) 0.06					
TG	40 (40%)	29 (34.9%)	35 (35.4%)										
TT	4 (4%)	0 (0%)	0 (0%)										

							0.46						
G	152 (76%)	137 (82. 5%)	163 (82. 3%)			0.67 (0.39 - 1.16) 0.16	1.00 (0.55 - 1.75) 1.00	0.68 (0.41 - 1.14) 0.14					

Table 4: The genotype and allele frequencies of eNOS VNTR in the studied groups.

	Control:	**DR**	**DNR**	**PDR**	**NPDR**	**DR vs. Control:**	**DR vs. DNR**	**DNR vs. Control:**	**PDR vs. Control:**	**PDR vs. DNR**	**NPDR vs. Control:**	**NPDR vs. DNR**	**PDR vs. NPDR**
Overall	**N=200**	**N=196**	**N=200**	**N=78**	**N=99**	OR (95% CI)	OR (95% CI)	OR (95% CI)	OR (95% CI)	OR (95% CI)	OR (95% CI)	OR (95% CI)	OR (95% CI)
bb	155 (77.5 %)	148 (75. 5%)	151 (75. 5%)	59 (75. 7%)	73 (73. 7%)	DM: 1.12 (0.7-1.83) >0.05 RM: 1.48 (0.51 - 4.42) >0.05	DM: 0.99 (0.62 -1.62) >0.05 RM: 3.53 (0.9-16.4) 0.05	DM: 1.2 (0.69 - 1.82) >0.05 RM: 0.42 (0.09 - 1.83) >0.05	DM: 1.11 (0.57 - 2.14) >0.05 RM: 1.50 (0.36 - 5.90) >0.05	DM: 0.99 (0.52 - 1.90) >0.05 RM: 3.60 (0.65 - 20.5) >0.05	DM: 1.23 (0.68 - 2.22) >0.05 RM: 1.47 (0.39 - 5.32) >0.05	DM: 1.10 (0.61 - 1.97) >0.05 RM: 3.5 (0.71 - 18.90) >0.05	DM: 0.90 (0.43 - 1.90) >0.05 RM: 1.02 (0.22 - 4.56) >0.05
~~ba~~	38 (19%)	38 (19. 4%)	46 (23 %)	15 (19. 2%)	21 (21. 2%)								
~~aa~~	7 (3.5 %)	10 (5.1 %)	3 (1.5 %)	4 (5.1 %)	5 (5.1 %)								
b	348 (87%)	334 (85. 2%)	348 (86. 4%)	133 (85. 3%)	167 (84. 3%)	1.16 (0.8-1.8) >0.05	1.09 (0.7-1.7) >0.05	1.06 (0.7-1.6) >0.05	1.16 (0.66 - 2.03) >0.05	1.16 (0.66 - 2.03) >0.05	1.24 (0.75 - 2.06) >0.05	1.24 (0.75 - 2.06) >0.05	0.93 (0.50 - 1.74) >0.05
a	52 (13%)	58 (14. 8%)	55 (13. 6%)	23 (14. 7%)	31 (15. 7%)								
Males	**N=100**	**N=99**	**N=98**										
bb	79 (79%)	69 (69. 7%)	77 (78. 6%)			DM: 1.6 (0.82 -3.3) >0.05 RM: 1.55 (0.37 -6.8) >0.05	DM: 1.6 (0.8-3.2) >0.05 RM: 3.1 (0.55 - 22.8) >0.05	DM: 1.03 (0.5-2.1) >0.05 RM: 0.50 (0.06-3.3) >0.05					
~~ba~~	17 (17%)	24 (24. 2%)	19 (19. 4%)										
~~aa~~	4 (4%)	6 (6.1 %)	2 (2%)										
b	175 (87.5 %)	162 (81. 8%)	173 (88. 3%)			1.6 (0.9-2.8) >0.05	1.7 (0.9-3.05) >0.05	0.9 (0.5-1.8) >0.05					
a	25 (12.5 %)	36 (18. 2%)	23 (11. 7%)										
Females	**N=100**	**N=97**	**N=102**										
bb	76 (76%)	79 (81. 5%)	74 (72. 5%)			DM: 0.72 (0.34 - 1.51) >0.05 RM: 1.4 (0.3-8.1) >0.05	DM: 0.60 (0.31 .24) >0.05 RM: 4.34 (0.45 - 103.9) >0.05	DM: 1.2 (0.61 -2.4) >0.05 RM: 0.32 (0.01 -3.5) >0.05					
~~ba~~	21 (21%)	~~14~~ (14. 4%)	27 (26. 5%)										
~~aa~~	3 (3%)	4 (4.1 %)	1 (1%)										
b	173 (86.5 %)	172 (88. 7%)	175 (85. 8%)			0.82 (0.43 -1.6) >0.05	0.8 (0.4-1.5) >0.05	1.06 (0.6-1.9) >0.05					
a	27 (13.5 %)	22 (11. 3%)	29 (14. 2%)										

5.1. *eNOS* Bioinformatic Prediction

The variation in exon 7 is from aspartic acid (D) to glutamic acid (E) at position 298 of the protein sequence. The bioinformatic analysis showed that the variant residue E is bigger than the wild-type residue D (Fig. 1). The

variant residue is located within a stretch of residues that interact with NOSIP therefore E can disturb the interaction and consequently its function. As per in silico prediction the variant is less pathogenic in fact predicted to be benign based on MetaRNN score (0.037). In addition, the wild-type residue is not conserved at this position thus suggesting variant is not damaging for the protein's structure and function.

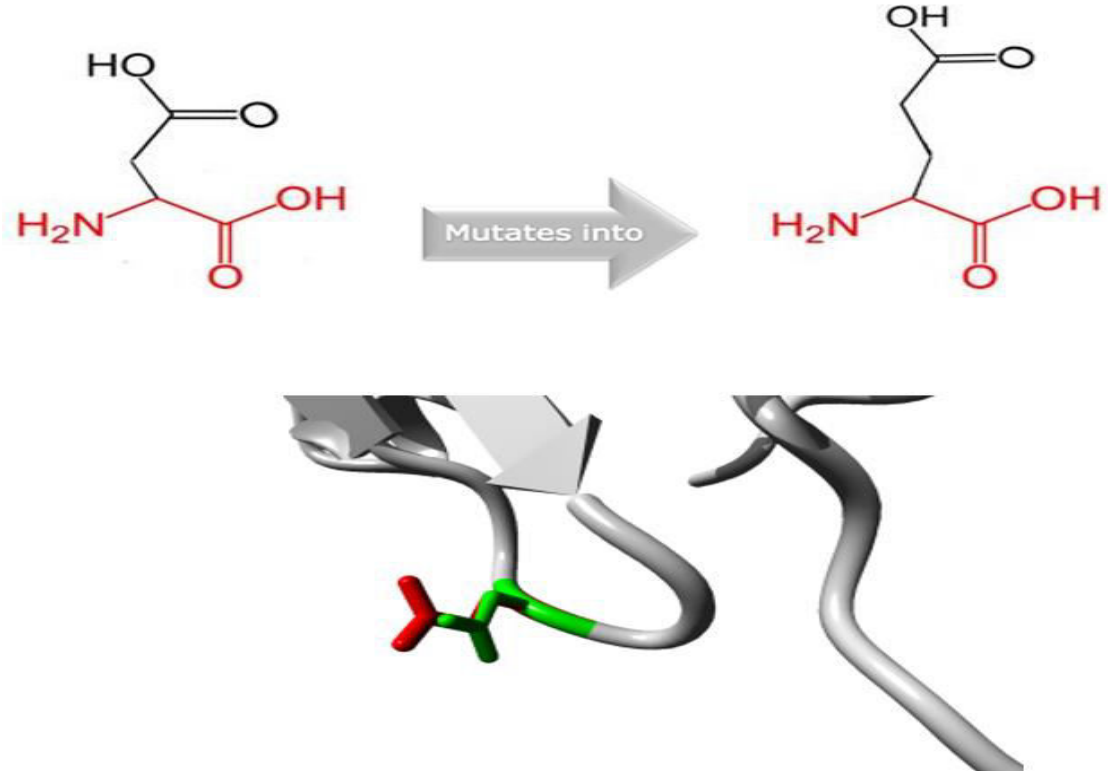

Figure 1: The bioinformatic analysis of *eNOS* variant rs1799983. In the figure the upper panel shows the schematic structures of the original (left) and the variant (right) amino acid. The backbone, which is the same for each amino acid, is colored red. The side chain, unique for each amino acid, is colored black. In the lower panel is the Close-up of the mutation. The protein is colored grey, the side chains of both the wild-type and the mutant residue are shown and colored green and red respectively.

Discussion

Current study hypothesized that oxidative stress and inflammatory pathways may be involved in the pathogenesis of diabetic retinopathy in southeast Asian countries. We set out current study to investigate the genetic polymorphism of inflammatory pathway markers like Heat Shock Protein (HSP 70) and endothelial nitric oxide (eNOS) genes in diabetic retinopathy patients in Pakistani population. Current study also set out to investigate the prevalence of DR in male and female Pakistani population. Current study came up with novel findings that polymorphism of HSP70 is found to be associated with diabetes retinopathy while, *eNOS* seemed to have no role or mild role in diabetic retinopathy susceptibility in south Asian diabetic patients. Current study for the first investigated that pathophysiology of diabetic retinopathy is gender specific and more prevalent in males as compared to females. Bioinformatic analysis also support the genetic analysis which conclude milder association of exonic SNP rs1799983 of eNOS with diabetic retinopathy which is not deleterious.

Current study has observed the frequency of GG, GC and CC genotypes DR patients for HSP 70 was 34.7%, 40.6% and 24.6%, respectively, with significant association found for the genotype distribution in the DR patients, as compared to the controls (χ^2=20.07, $p<0.05$). This indicates a strong link between HSP 70 genetical polymorphism and diabetic retinopathy. Similar association was also reported in Pakistani population between HSP 70 and Primary Closed Angle Glaucoma (PCAG) [37] where diminished protective response of HSP 70 was observed due to increased stress level in the eye. In current study The -27 (G/C) polymorphism of *HSP70* was thus chosen for genetic analysis, as this polymorphism has not been previously investigated for its involvement with DR. Functional analyses of the -27 (G/C) polymorphism, which maps to the 5′ UTR region of *HSP70*, have previously revealed that compared with the G allele, the variant C allele results in reduced promoter activity and lower *HSP70* protein levels [38]. In glaucoma study on Pakistani population, a link between PCOG and G+190C of HSP 70 polymorphism was established [37] while other study reported [39]

reported no association between A-110C polymorphism of HSP 70-1 and Primary Open Angle Glaucoma (POAG) in Japanese population which indicates same gene polymorphism may be responsible for one disease in one population but no link with the same disease in other population. Not only HSP 70, another member of this family HSP 29 was elevated in the aqueous humor of patient with acute primary angle glaucoma [40]. This point of variation of genetic polymorphism of HSP 70 and its association with DR susceptibility was the baseline hypothesis for southeast Asian countries like Pakistan.

Heat shock protein 70 is considered as pathway of pathogenesis in streptozotocin model of diabetes mellitus (DM) by upregulation of HSP70/HSP90/TLR4/AMPK signaling pathway (2) while HSP 70 was elevated in response to heat in the retina of chicken (3) while HSP 70 and 90 are reported to be elevated in Pakistani sheep breed [41]. Similarly, HSP 70 is also involved in the pathogenesis of diabetic nephropathy [42] which clearly indicates that HSP 70 should be considered as an early marker of the disease rather than a protective gene involved as a barrier in the pathology. It is point of contention that HSP 70 provide resistance in the onset of the disease but at the same time HSP 70 levels are further elevated in the pathological situation which nullify the notion that HSP 70 has a protective role as mentioned in the study reported [43] that geranylgeranylacetone (GGA) induced HSP70 overexpression has protective effects on retinal I/R injury by activating PI3K/AKT/mTOR signaling. Oxidative stress and inflammatory markers are elevated in diabetic retinopathy [44] which consequently increases the levels of interleukin-1β (IL-1β), IL-6, IL-8, IL-17A and tumor necrosis factor-α (TNF-α) which was reported to have a positive relation between the expression levels of interleukin and manifestations of DR[45, 46]. It is certain that in response to oxidative stress the expression of these inflammatory cytokines may be temporary arrested by HSP 70 as reported [47] but at the same time disease progress along with elevation of HSP 70 expression in retina. Levels of HSP 70 begin to be elevated with the onset of DM [15, 42] and when complications of DM start to manifest in the form of diabetic retinopathy HSP 70 levels reach to its maximum limit. One member of HSP family is huperzine A is reported to be a potential therapeutic drug to prevent diabetic retinopathy in pharmacological model. Huperzine A is a natural alkaloid isolated from Huperzia serrata, displays neuroprotective and antiapoptotic effects in treating neurodegenerative disorders [48]. Heat shock protein 70 enhance the complication of the eye by deposition of collagen in the eye. Data reported that inhibition of HSP 70 inhibited the collagen deposition [49] which indicates that progression of DR can be arrested by using natural alkaloid like Huperzine A. Observations of current stands unique in the sense that none of the study identified the pathological association of HSP 70 genetic polymorphism with the diabetic retinopathy.

Current study for the first investigated that pathophysiology of diabetic retinopathy is gender specific and more prevalent in males as compared to females.As far as the gender differences are concerned, it has previously been reported that due to estrogen effect, females have higher basal levels of HSPs as compared to males [50]. This statement is in agreement with the findings of current study where the males are highly susceptible to DR with a higher preponderance of the C risk allele in DR cases as compared to controls but female allele distribution does not seem to be associated with the disease. Studies have revealed strong antioxidant properties of estrogen [51]. Studies on the female mice versus male mice[52] reported that female mice were resistant to the onset of diabetes and its complication with same dose used for male mice strongly correlated with the findings of current study in the Pakistani population. Gender seems to play and important role in the onset of diabetes and diabetic retinopathy which is also reported in the data published [53] on male-female differences in diabetic retinopathy. Incidence of diabetic retinopathy is more prevalent in males [54] which suggested neither serum testosterone nor other sex hormones were related to the incidence of severe diabetic retinopathy. Current study for the first investigated that pathophysiology of diabetic retinopathy is gender specific and more prevalent in males as compared to females.

Although studies on glaucoma in Pakistani population showed the eNOS gene polymorphism association with the disease [34] but current study showed that eNOS gene polymorphism showed no association with DR. The rs1799983 (c.894G>T; p.Glu298Asp), a non-synonymous SNP found in exon 7 of *eNOS* was also screened in the current study. Its two alleles have been reported to be associated with different NO levels. The individuals with the mutant T allele have been known to have lower NO synthesis. In this variant at the protein level, glutamate (Glu) residue is replaced with aspartate (Asp), which makes the protein more vulnerable to degradation or proteolytic cleavage, which leads to reduced NO levels, hence disrupting proper endothelial function [54]. GG genotype was the most frequently found in the three groups, while the TT genotype was only rarely found in controls and it was completely absent in cases (DR and DNR). The TT genotype might even be present in cases had the sample size been larger. Many significant associations found in the analysis of this SNP that were observed were probably due to the frequency distribution pattern, i.e. total lack of TT genotype in

cases. Therefore, it can be presumed that this non-synonymous SNP in *eNOS* does not have any role in altering disease susceptibility, or plays a minor role. The frequency of the SNP varies among different populations [55]. Similar type of data is reported in patients with diabetic nephropathy[56, 57] and retinopathy [58] and Chinese population with rheumatoid arthritis [59] while the findings is different in Indian population where the TT genotype is absent in controls and present in diabetic retinopathy cases in south indian population [60, 61]. In agreement to data of eNOS in DR patient in Indian population studies conducted on *eNOS* VNTR among Slovenian Caucasians demonstrated the association of eNOS "aa" genotype with the disease [62] showing this genotype as a stronger risk factor for the DR onset in Caucasians. The association of this genotype was also found with type 1 diabetic individuals [63]. However, in our study, we also found no association of the VNTR with the disease.

Bioinformatic analysis of exonic SNP rs1799983 of eNOS showed the milder effect on the diabetic retinopathy which is not deleterious, and findings are in cohesion with our genetic analysis part of the study.

Conclusion

Current study concluded that polymorphism of HSP70 is associated with diabetes retinopathy while, *eNOS* seemed to have no role in diabetes retinopathy susceptibility in south Asian diabetic patients. Current study for the first investigated that pathophysiology of diabetic retinopathy is gender specific and more prevalent in males as compared to females in Pakistani population. Bioinformatic analysis also support the genetic analysis which conclude milder association of exonic SNP rs1799983 of eNOS with diabetic retinopathy which is not deleterious.

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