Final Report of Major Research Project

(From 01/ 04/ 2013 to 31/ 03/ 2017)

"Association of Single Nucleotide Polymorphisms with Type 2 Diabetes and Diabetic Retinopathy in Western Indian Population"

Submitted To

University Grant Commission

Bahadur Shah Zafar Marg

New Delhi – 110002

Submitted By

Dr. (Mrs.) Kiran Kalia

Professor in Biochemistry

BRD School of Bioscience, Sardar Patel University,

Vallabh Vidyanagar – 388 120

Gujarat

Annexure - III

BRD School of Bioscience
Sardar Patel University
Vallabh Vidyanagar – 388 120.

STATEMENT OF EXPENDITURE IN RESPECT OF MAJOR RESEARCH PROJECT

1. Name of Principal Investigator: Dr. (Mrs.) Kiran Kalia

2. Deptt. of Principal Investigator: B R D School of Biosciences

   University/College: Sardar Patel University, Gujarat

3. UGC approval Letter No. and Date: F.-42-637/2013(SR) dated 22/03/2013

4. Title of the Research Project: **"**Association of Single Nucleotide Polymorphisms with Type 2 Diabetes and Diabetic Retinopathy in Western Indian Population"

5. Effective date of starting the project: 01/ 04/ 2013

6. a. Period of Expenditure: From 01/ 04/ 2013 to 31/ 3/2017

   b. Details of Expenditure:

S. No.	Item	Amount Approved (Rs.)	Total Amount Received (Rs.)	Expenditure Incurred (Rs.)				Total Expenditure Incurred (Rs.)
				2013-14	2014-15	2015-16	2016-17	
i.	Books & Journals	10,000/-	10,000/-	Nil	Nil	Nil	Nil	Nil
ii.	Equipment	1,50,000/-	1,50,000/-	1,50,000/-	Nil	Nil	Nil	1,50,000/-
iii.	Contingency	1,50,000/-	1,35,000/-	60,392/-	29,655/-	46,177/-	13,776/-	1,50,000/-
iv.	Field Work/Travel (Give details in the Performa at Annexure-IV)	30,000/-	27,000/-	2,872/-	10,048/-	15,417/-	1,663/-	30,000/-
v.	Hiring Services	Nil	Nil	Nil	Nil	Nil	Nil	Nil
vi.	Chemicals & Glassware	4,00,000/-	3,60,000/-	2,04,577/-	1,29,297	20,589/-	45,536/-	3,99,999/-
vii.	Overhead	1,07,800/-	1,07,800/-	1,07,800/-	Nil	Nil	Nil	1,07,800/-
viii.	Any other items (Please specify)	Nil	Nil	Nil	Nil	Nil	Nil	Nil

c. Staff

Date of Appointment: 01/07/2013

S.No	Item	From	To	Amount Approved (Rs.)	Amount Received (Rs.)	Expenditure Incurred (2015-2017) (Rs.)	Total Expenditure (Rs.)
1.	Honorarium to PI (Retired Teachers) @ Rs. 18,000/-p.m.	NA	NA	NA	NA	NA	
2.	**Project fellow:** i) **NET/GATE qualified-**Rs. 16,000/- p.m. for initial 2 years and Rs. 18,000/- p.m. for the third year. ii) **Non-GATE/ Non-NET-** Rs. 14,000/-p.m. for initial 2 years and Rs. 16,000/-p.m. for the third year.	1/07/2013	31/3/2016	5,28,000/-	4,32,000/-	2,48,000/-	5,28,000/-

1. It is certified that the appointment(s) have been made in accordance with the terms and conditions laid down by the Commission.
2. If as a result of check or audit objection some irregularly is noticed at later date, action will be taken to refund, adjust or regularize the objected amounts.
3. Payment @ revised rates shall be made with arrears on the availability of additional funds.
4. It is certified that the grant of **Rs. 12,21,800/- (Twelve Lac Twenty One Thousand Eight Hundred only)** received from the University Grants Commission under the scheme of support for Major Research Project entitled **"Association of Single Nucleotide Polymorphisms with Type 2 Diabetes and Diabetic Retinopathy in Western Indian Population"** of **Dr. Kiran Kalia,** B R Doshi School of Biosciences, vide UGC letter No. **F.-42-637/2013(SR) dated 22/03/2013. Out of this, an amount of Rs. 13,65,799 /- (Thirteen Lac Sixty Five Thousand Seven Hundred Ninety Nine only**) has been utilized for the purpose for which it was sanctioned and in accordance with the terms and conditions laid down by the University Grants Commission, New Delhi.

PRINCIPAL INVESTIGATOR

Dr. (Mrs.) Kiran Kalia
Principal Investig.

SARDAR PATEL UNIVERSITY

RHRajput
12.4.17

REGISTRAR

Registrar
Sardar Patel University
Vallabh Vidyanagar.

Annexure - IV

BRD School of Bioscience
Sardar Patel University
Vallabh Vidyanagar – 388 120

STATEMENT OF EXPENDITURE INCURRED ON FIELD WORK

Name of the Principal Investigator: Dr. (Mrs.) Kiran Kalia

2013-2014

Name of the Place visited		Date of the Visit	Mode of Journey	Expenditure Incurred (Rs.)
From	**To**			
Dr. Sarita Gupta From M S University, Baroda to BRD School of Biosciences, Vallabh vidyanagar (For TA/DA of Project fellow interview)		22/ 06/ 2013	By Road (Car)	980/-
BRD School of Biosciences, Vallabh vidyanagar	P S Medical College, Karamsad	12/10/2013	By Road (Auto)	1892/- Including Return to BRD School of Biosciences, Vallabh vidyanagar
BRD School of Biosciences, Vallabh vidyanagar	P S Medical College, Karamsad	19/10/2013		
BRD School of Biosciences, Vallabh vidyanagar	Santram Eye hospital, Nadiad	26/10/2013		
BRD School of Biosciences, Vallabh vidyanagar	Santram Eye hospital	9/11/2013		
BRD School of Biosciences, Vallabh vidyanagar	P S Medical College, Karamsad.	16/11/2013		
BRD School of Biosciences, Vallabh vidyanagar	Santram Eye hospital, Nadiad	23/11/2013		
BRD School of Biosciences, Vallabh vidyanagar	Santram Eye hospital, Nadiad	30/11/2013		
BRD School of Biosciences, Vallabh vidyanagar	P S Medical College, Karamsad	7/12/2013		
BRD School of Biosciences, Vallabh vidyanagar	P S Medical College, Karamsad	14/12/2013		
BRD School of Biosciences, Vallabh vidyanagar	Santram Eye hospital, Nadiad	21/12/2013		
BRD School of Biosciences, Vallabh vidyanagar	Santram Eye hospital, Nadiad	28/12/2013		

2014-2015

Name of the Place visited		Date of the Visit	Mode of Journey	Expenditure Incurred (Rs.)
From	**To**			
BRD School of Biosciences, Vallabh vidyanagar	Santram Eye hospital, Nadiad	04/ 01/ 2014 11/ 01/ 2014 18/ 01/ 2014 25/ 01/ 2014 01/ 02/ 2014 08/ 02/ 2014 15/ 02/ 2014 22/ 02/ 2014 01/ 03/ 2014 08/ 03/ 2014 15/ 03/ 2014 22/ 03/ 2014 29/ 03/ 2014	By Road (Auto)	**2,236/** Including return to BRD School of Biosciences, Vallabh vidyanagar
BRD School of Biosciences, Vallabh vidyanagar	P S Medical College, Karamsad. .	16/ 04/ 2014 21/ 04/ 2014 23/ 04/ 2014 30/ 04/ 2014 7/ 05/ 2014 14/ 05/ 2014 19/ 05/ 2014 21/ 05/ 2014 28/ 05/ 2014 02/ 06/ 2014 04/ 06/ 2014 11/ 06/ 2014 18/ 06/ 2014 09/ 07/ 2014 16/ 07/ 2014 23/ 07/ 2014 30/ 07/ 2014 06/ 08/ 2014 13/ 08/ 2014 20/ 08/ 2014 27/ 08/ 2014 03/ 09/ 2014 10/ 09/ 2014 17/ 09/ 2014 29/ 09/ 2014	By Road (Auto)	**2,400/-** Including return to BRD School of Biosciences, Vallabh vidyanagar
BRD School of Biosciences, Vallabh vidyanagar	Santram Eye hospital, Nadiad	12/ 04/ 2014 26/ 04/ 2014 10/ 05/ 2014 31/ 05/ 2014 14/ 06/ 2014	By Road	**1,548/-** Including return to BRD School of

		12/ 07/ 2014 02/ 08/ 2014 06/ 09/ 2014 27/ 09/ 2014	(Auto)	Biosciences, Vallabh vidyanagar
BRD School of Biosciences, Vallabh vidyanagar	P S Medical College, Karamsad.	08/10/2014 13/10/2014 15/10/2014 29/10/2014 3/11/2014 5/11/2014 10/11/2014 12/11/2014 19/11/2014 24/11/2014 1/12/2014 3/12/2014 8/12/2014 15/12/2014 5/01/2015 7/01/2015 19/01/2015 21/01/2015	By Road (Auto)	**1,800/-** Including return to BRD School of Biosciences, Vallabh vidyanagar
BRD School of Biosciences, Vallabh vidyanagar	Santram Eye hospital, Nadiad	11/10/2014 18/10/2014 1/11/2014 8/11/2014 15/11/2014 22/11/2014 6/12/2014 13/12/2014 20/12/2014 3/1/2015 10/1/2015 24/1/2015 7/2/2015 14/2/2015 21/2/2015 28/2/2015 7/2/2015 14/2/2015	By Road (Auto)	**2064/-** Including return to BRD School of Biosciences, Vallabh vidyanagar

2015-2016

Name of the Place visited		Date of the Visit	Mode of Journey	Expenditure Incurred (Rs.)
From	**To**			
BRD School of Biosciences, Vallabh vidyanagar	P S Medical College, Karamsad	28/ 01/ 2015	By Road (Auto)	**2,704/-** Including return to BRD School of Biosciences, Vallabh vidyanagar
		02/ 02/ 2015		
		04/ 02/ 2015		
		09/ 02/ 2015		
		11/ 02/ 2015		
		16/ 02/ 2015		
		18/ 02/ 2015		
		23/ 02/ 2015		
		02/ 03/ 2015		
		04/ 03/ 2015		
		09/ 03/ 2015		
		11/ 03/ 2015		
		16/ 03/ 2015		
		18/ 03/ 2015		
		23/ 03/ 2015		
	Santram Eye hospital, Nadiad	31/ 01/ 2015		
		07/ 02/ 2015		
		14/ 02/ 2015		
		21/ 02/ 2015		
		28/ 02/ 2015		
		07/ 03/ 2015		
		14/ 03/ 2015		
BRD School of Biosciences, Vallabh vidyanagar	P S Medical College, Karamsad	27/ 04/ 2015	By Road (Auto)	
		29/ 04/ 2015		
		04/ 05/ 2015		
		06/ 05/ 2015		
		13/ 05/ 2015		
		18/ 05/ 2015		
		27/ 05/ 2015		
		28/ 05/ 2015		
		1/ 06/ 2015		
		10/ 06/ 2015		
		15/ 06/ 2015		
		17/ 06/ 2015		
		08/ 07/ 2015		
		13/ 07/ 2015		

BRD School of Biosciences, Vallabh vidyanagar		15/ 07/ 2015		**4,190/-** Including return to BRD School of Biosciences, Vallabh vidyanagar
		22/ 07/ 2015		
	P S Medical College, Karamsad	29/ 07/ 2015		
		05/ 08/ 2015		
		12/ 08/ 2015		
		27/ 08/ 2015		
		02/ 09/ 2015		
		09/ 09/ 2015		
		14/ 09/ 2015		
		23/ 09/ 2015		
		30/ 09/ 2015		
	Santram Eye hospital, Nadiad	02/ 05/ 2015	By Road (Auto)	
		16/ 05/ 2015		
		23/ 05/ 2015		
		30/ 05/ 2015		
		20/ 06/ 2015		
		04/ 07/ 2015		
		11/ 07/ 2015		
		25/ 07/ 2015		
		01/ 08/ 2015		
		08/ 08/ 2015		
		22/ 08/ 2015		
		12/ 09/ 2015		
		19/ 09/ 2015		
BRD School of Biosciences, Vallabh vidyanagar	Civil Hospital, Ahmedabad	2/ 06/ 2015	By Road (auto and bus), Train	5,745/- Including return to BRD School of Biosciences, Vallabh vidyanagar
		06/ 06/ 2015		
		22/ 06/ 2015		
		23/ 06/ 2015		
		01/ 07/ 2015		
		09/ 07/ 2015		
		15/ 07/ 2015		
		20/ 10/ 2015		
		29/10/2015		
		3/11/2015		
		15/ 11/ 2015		
		16/ 11/ 2015		
		30/11/15		
BRD School of Biosciences, Vallabh	P S Medical College,	02/ 11/ 2015		
		05/ 11/ 2015		
		18/ 11/ 2015		
		19/ 11/ 2015		

	Karamsad.	23/ 11/ 2015 26/ 11/ 2015 02/ 12/ 2015 03/ 12/ 2015 07/ 12/ 2015 09/ 12/ 2015 14/ 12/ 2015 16/ 12/ 2015 21/ 12/ 2015 23/ 12/ 2015 28/ 12/ 2015 30/ 12/ 2015	By Road (Auto)	**2,778/-** Including return to BRD School of Biosciences, Vallabh vidyanagar
BRD School of Biosciences, Vallabh vidyanagar	Santram Eye hospital, Nadiad	07/ 11/ 2015 21/ 11/ 2015 05/ 12/ 2015 26/ 11/ 2015 19/ 12/ 2015 26/ 12/ 2015		
BRD School of Biosciences, Vallabh vidyanagar	P S Medical College, Karamsad.	04/ 01/ 2016 06/ 01/ 2016 11/ 01/ 2016 18/ 01/ 2016 20/ 01/ 2016 25/ 01/ 2016 27/ 01/ 2016 01/ 02/ 2016 03/ 02/ 2016	By Road (Auto)	**1,663/-** Including return to BRD School of Biosciences, Vallabh vidyanagar
BRD School of Biosciences, Vallabh vidyanagar	Santram Eye hospital, Nadiad	02/ 01/ 2016 09/ 01/ 2016 23/ 01/ 2016 30/ 01/ 2016		
Total				10,000/-

Certified that the above expenditure is in accordance with the UGC norms for Major Research Projects

PRINCIPAL INVESTIGATOR

Dr. (Mrs.) Kiran Kalia
Principal Investigator

REGISTRAR

RHRajpu 12.4.17
Registrar
Sardar Patel University
Vallabh Vidyanagar.

Encl. 01

Dr. (Mrs.) Kiran Kalia
Professor in Biochemistry

B R DOSHI SCHOOL OF BIOSCIENCES
Sardar Patel Maidan, Vadtal Road
Post Box No. 39
SARDAR PATEL UNIVERSITY
Vallabh Vidyanagar 388 120 (Gujarat) India
Tel. : +91-2692-234402 / 234412 / 231042
Fax : +91-2692-231041 / 236475
Website : www.spuvvn.edu/pgd/biosciences

PGB/KK/215

Annexure-IX

UGC File No. F.-47-637/2013(SR) **YEAR OF COMMENCEMENT** 0 1 | 0 4 | 2 0 1 3
Dated: 22/03/2013

TITLE OF THE PROJECT: Association of Single Nucleotide Polymorphisms with type 2 diabetes and diabetic retinopathy in Western Indian population

1.	Name of the Principle Investigator	Prof. Kiran Kalia				
2.	Name of the University/ College	Sardar Patel University				
3.	Name of the Research Personnel appointed	Dhara N. Jajal				
4.	Academic Qualification	S. No.	Qualifications	Year	Marks	Percentage
		1.	M.Sc. Biochemistry	2011	1522/2200	69.2%
5.	Date of Joining	01/ 07/ 2013				
6.	Date of Birth of Research Personnel	04/ 10/ 1988				
7.	Amount of HRA, if drawn	———				
8.	Number of Candidate applied for the post	17 applied, 15 appeared for interview				

CERTIFICATE

This is to certify that all the rules and regulations of UGC Major Research Project outlined in the guidelines have been followed. Any lapses on the part of the University will liable to terminate of said UGC project.

Principle Investigator

Head of the Dept.

I/Registrar
Sardar Patel University
Vallabh Vidyanagar

Annexure - VIII

BRD School of Bioscience
Sardar Patel University
Vallabh Vidyanagar – 388 120.

Final Report of the work done on the Major Research Project

1. **Project report No.:** Final

2. **UGC Reference No.:** F.-42-637/2013(SR) dated 22/03/2013

3. **Period of report:** From 01/ 04/ 2013 to 31/ 03/ 2017

4. **Title of research project:** **"**Association of Single Nucleotide Polymorphisms with Type 2 Diabetes and Diabetic Retinopathy in Western Indian Population"

5. (a) **Name of the Principal Investigator:** Prof. (Mrs.) Kiran Kalia
   (b) **Deptt.:** B.R.D. School of Biosciences
   (c) **University/College where work has progressed**: B.R.D. School of Biosciences, Sardar Patel University, Vallabh Vidyanagar, Gujarat.

6. **Effective date of starting of the project:** 01/ 04/ 2013

7. **Grant approved and expenditure incurred during the period of the report:**
   a. **Total amount approved:** Rs. 13, 75, 800/-
   b. **Total amount received up to 10/ 04/ 2017:** Rs.12,21,800 /-
   c. **Total expenditure:** Rs.13,65,799 /-
   d. **Balance (Receivable):** **(-)** Rs. 1,43,999/-
   e. **Report of the work done:** (Please attach a separate sheet: Encl. 1)

Encl. 1

Report of the Work Done

i. Brief objective of the project

Type 2 diabetes mellitus (T2D) is one of the major subtypes of diabetes accounts for more than 95% of all diabetes. Persistent diabetes leads to various secondary complications that are responsible for the morbidity and mortality. Diabetic retinopathy (DR) is the most frequently observed microvascular complication of diabetes and the leading cause of preventable blindness. By 2025, it is estimated that 40% of the total diabetic patients would have some form of DR (Kempen et al, 2004).

Previous studies have evidently shown the ethnicity-specific high prevalence of DR and its heritability in different populations including an Indians. Thus, genetic factors are well established in implicating predisposition to the development and progression of DR as well. The identification of genetic risk factors is an area of substantial research for developing screening algorithms for early detection of DR or T2D. One of the common approaches for performing such genetic association studies is candidate gene approach, where several genes encoding proteins closely related to the disease is analyzed in case-control studies.

Vascular endothelial growth factor (VEGF), a key multifunctional mitogen, is a potent mediator of angiogenesis and microvascular permeability. Genetic variations in VEGF gene induce hyperpermeability of retinal vessels, breakdown of the blood–retinal barrier and neovascularization, leading to the development of DR (Schlingemann and van Hinsbergh, 1997). The polymorphisms reported in the promoter, 5’ UTR and 3’ UTR of VEGF gene showed strong association with diabetic retinopathy in Chinese, Caucasian, and Southern part of an Indian population (Yang et al, 2011; Ray et al, 2004 and Suganthalakshmi et al, 2006). But they need to be validated in the western part of India.

Calpain 10 (CAPN10), a member of the calcium-activated intracellular proteases. The increased glucose concentration can alter the charges across the cell membranes and stimulate Ca+2 channels to increase Ca+2influx of the cell. The increased intracellular Ca+2 concentrations activate Calpain 10, which ultimately found to cause apoptotic changes in pancreatic β- cell leading to its destruction (Zhou et al, 2003). It has been suggested that polymorphisms of CAPN10 gene may impair glucose-induced insulin secretion in the pancreatic β cell and glucose uptake in skeletal muscle and adipocytes (Turner et al, 2005; Zhou et al, 2003). Thus, we propose to study

the most studied SNPs of CAPN 10 gene in various populations and need to validate in our population with type 2 diabetes and diabetic retinopathy.

Hence, we aimed to study following SNPs in VEGF gene in association with type 2 diabetes and diabetic retinopathy in western part of India.

- rs699947 (-2578 C>A), rs833061 (-1498 T>C), rs13207351 (-1190 G>A) of promoter region; rs2010963 (-634 C>G) of 5' UTR; rs833069 (3596 A>G), rs2146323 (6112 C>A) of intron 2; rs3025021 (10180 T>C) of intron 6; rs3025039 (13553 C>T) of 3' UTR.
- To study association of SNP19 (rs3842570), SNP43 (rs3792267) and SNP63 (rs5030952) of CAPN10 gene with type 2 diabetes and diabetic retinopathy in western part of Indian population.

ii. Work done so far and results achieved and publications, if any, resulting from the work (Give details of the papers and names of the journals in which it has been published or accepted for publication)

Sample Collection

The present study is ethically approved by Human Research Ethics Committee of P.S. Medical College and hospital, Karamsad. The blood and urine samples of normal healthy individuals and patients visiting P.S. Medical College and hospital, Karamsad were collected in EDTA coated vacutainers and urine sample storage vials respectively. The current study recruited 258 patients with type 2 diabetes attending Pramukh Swami Medical College and Hospital, Karamsad, Gujarat. During the same period, a group of 93 healthy individuals was enrolled in the study as healthy controls. They were volunteers, blood donors, or relatives to the patients that visited the hospital. The treating physician diagnosed the patients with type 2 diabetes (T2D) as per the American Diabetes Association (ADA) guidelines. All the patients and control subjects were undergone visual acuity and fundus examination through dilated pupils. An expert ophthalmologist diagnosed them for DR grading according to Diabetic Retinopathy Disease Severity Scale that was based on Early Treatment Diabetic Retinopathy Study (ETDRS). Informed consent of all the patients was obtained and history of the patients along with their Clinical parameters like age, sex, BMI, blood pressure was noted.

The samples collected are categorized in following groups:

1. **HC** **Control healthy individual (93)**
2. **DWR** **Type 2 Diabetic patients without any complications(110)**
3. **DR** **Type 2 Diabetic patients with Retinopathy(148)**
4. **PDR - subgroup of DR** **Type 2 Diabetic patients with Proliferative Retinopathy(62)**

Numbers in the brackets are showing no. of samples collected till date.

Biochemical parameters like Fasting blood glucose (FBG), Glycated haemoglobin (GHb), Serum creatinine (S.Cr.) were done by GOD-POD method, TBA method and by Jaffe's Method respectively.

Isolation of genomic DNA from the blood cells of all samples was done using Qiagen DNA isolating kit; followed by its quantitative and qualitative analysis. The DNA bank of all the samples is stored at -20°C in TE buffer.

Genotyping of the SNPs was done by performing PCR/ RFLP, Direct Sequencing and Next Generation Sequencing.

Primer Designing

Primers were designed for amplification of specific regions in VEGF using human reference sequences from NCBI gene database and primer 3 (input version). We have used reported primers to study three SNPs of Calpain 10 (*Evans et al, 2001*). All the primers used to amplify the specific regions of VEGF and CAPN 10 genes, and their product sizes are given in table 1 and 2 respectively.

Genotyping of SNPs

All the regions of VEGF gene containing the targeted SNPs were amplified in the 25 µl reaction volume using NEB Taq 2x master mix, the specific primers (given in table 3) and genomic DNA in ABS Verity thermocycler. The cycling conditions for all the PCR reactions were optimized and Tm of the reactions were determined by performing gradient PCR. The amplified products were verified on the 1.8% agarose gel for its specific amplification and further used for restriction digestion.

Table 1: Primers used to genotype the SNPs of VEGF

SNP	Forward Primer	Reverse Primer	Product size (bp)
rs699947 (-2578 C / A)	5'CCCTTTTCCTCCAACTCTCC3'	5'CATCCTCAGCACATGTTGCT3'	311
rs833069 (3596 A/G)	5'GTTCACAGCACCCGAACATA3'	5'GAACAGCGGAGAGTCCTCAC3'	358
rs2146323 (6112 C/A)	5'GTCTCGATTGGATGGCAGTA3'	5'CCCATACTCAGACTGTCCTCT3'	384
rs3025021 (10180 T/C)	5'TTCCACCAAGGTGGGCTAAA3'	5'CTGCTCACCCAACTGGTTTC3'	352
rs3025039 (13553 C/T)	5'CCTCCCAACTCAAGTCCACA3'	5'CACCATCGACAGAACAGTCC3'	395

Table 2: Primers used to genotype the SNPs of Calpain 10

SNP	Forward Primer	Reverse Primer	Product size (bp)
SNP-19 rs3842570	5'GTTTGGTTCTCTTCAGCGTGGAG3'	5'CATGAACCCTGGCAGGGTCTAAG3'	187 for insertion, otherwise 155
SNP-43 rs3792267 SNP-44 rs2975760	5'GATGTGGGCATCCATAGCTTC3'	5'AAAAGCTACAGTGTGCCTGAG3'	593

Restriction enzymes for detection of particular SNPs in VEGF gene were designed by WEB Cutter 2.0 which was verified by the NEB cutter. The amplified products for all the studied SNPs were subjected to restriction digestion using their specific restriction enzymes at 37°C for overnight. The digested products were run on 2.8% agarose gel for determining the band pattern and subsequently genotyped for the particular SNP using the band pattern given in table 3.

SNP-19 of CAPN 10: This insertion polymorphisms was identified by separating PCR products on 2.8% agarose gel electrophoresis: allele I (three repeats of 32-bp sequence) is of 187 bp and allele – (two repeats of 32-bp sequence) is of 155 bp.

Table 3: Restriction enzymes and their band pattern used to Genotype SNPs of VEGF gene

SNP	Restriction Enzyme	Genotype	Band Size (bp)
rs699947 (-2578 C / A)	Bgl II	C/C	311
		C/A	311, 186 & 125
		A/A	186 & 125
rs833069 (3596A/G)	BseRI	A/A	274 & 84
		A/G	358, 274 & 84
		G/G	358
rs2146323 (6112 C/A)	MluCI	C/C	384
		C/A	384, 224 & 160
		A/A	224 & 160
rs3025021 (10180 T/C)	NciI	T/T	19, 333
		T/C	19, 333, 260 & 73
		C/C	19, 260 & 73
rs3025039 (13553 C/T)	NlaIII	C/C	395
		C/T	395, 272 & 123
		T/T	272 & 123

SNP-43 and SNP - 44 of CAPN 10: This SNP was genotyped by PCR- Direct Sequencing and used primers showed in Table 2. The PCR products were ran on a 2.8% agarose gel to verify 593bp product. All the electrogram sequences were analyzed for genotyping SNP-43 and -44 by mutation surveyor software version 5.1 followed by manual verification of the SNPs. We reconfirmed the presence of minor allele at polymorphic locus in randomly selected 10% of the samples by sequencing a PCR product derived independently from the original template.

rs833061 (-1498 T>C), rs13207351 (-1190 G>A) and **rs2010963 (-634 C>G)** were genotyped in 45 DWR and 55 DR Patients using Next generation sequencing on Illumina platform.

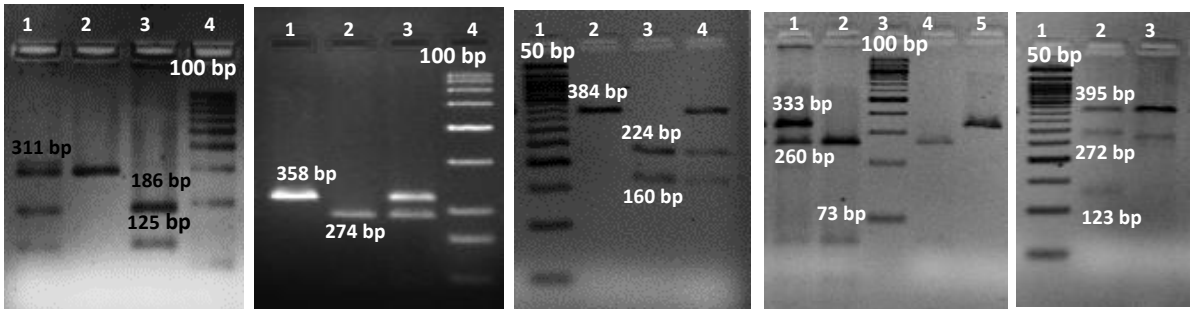
Results

The clinical characteristics of the study groups that include age, gender, BMI, duration of diabetes, genetic history and biochemical parameters of the diabetes are summarized in table 4.

Table 4: Clinical and biochemical characteristics of the study groups

Parameters	HC	DWR	DR	P value
Number	93	110	148	
Age (years)	48.3 ± 11.5	55.3 ± 9.0	59.1 ± 7.4	a*** b*** c**
Gender [n (%)]				
Male	53 (57.0)	66 (60.0)	88 (59.5)	$(a\ b\ c)^{NS}$
Female	40 (43.0)	44 (40.0)	60 (40.5)	
BMI (kg/m^2)	24.3 ± 3.9	26.4 ± 4.0	24.9 ± 4.0	a** b^{NS} c*
Hypertensive Patients [n (%)]		59 (53.6)	95 (64.2)	C^{NS}
SBP (mm hg)	122.0 ± 7.4	131.9 ± 16.4	132.7 ± 14.1	a*** b*** c^{NS}
DBP (mm hg)	79.5 ± 5.6	80.9 ± 9.2	82.6 ± 9.2	a^{NS} b* c^{NS}
Duration of diabetes (years)	-	7.5 ± 3.3	8.9 ± 5.4	C^{NS}
Unknown	-	0	7	
Hypoglycaemic agents [n (%)]				
Oral Hypoglycaemic agents	-	101 (91.8)	115 (77.7)	C*
Insulin	-	3 (2.7)	13 (8.8)	
Oral + Insulin	-	4 (3.7)	14 (9.5)	
No medications	-	2 (1.8)	6 (4.0)	
Family History of Diabetes [n (%)]		51 (49.0)	66 (46.2)	
Mother	-	29	29	C^{NS}
Father	-	25	20	
Siblings	-	15	35	
Unknown	-	6	5	
Habits [n (%)]		43 (39.1)	54 (36.5)	
Tobacco chewing/ sniffing	-	26/7	29/2	C^{NS}
Smoking	-	13	17	
Occasional/ former Habituate	-	3/0	8/2	
Fasting Plasma Glucose (mg/dl)	84.5 ± 7.2	130.2 ± 20.6	138.9 ± 26.4	a*** b*** c*
Glycated Hemoglobin (%)	6.31 ± 0.68	8.99 ± 0.71	9.57 ± 1.04	a*** b*** c***
Serum Creatinine (mg/dl)	0.99 ± 0.26	1.01 ± 0.28	1.12 ± 0.31	a^{NS} b** c*

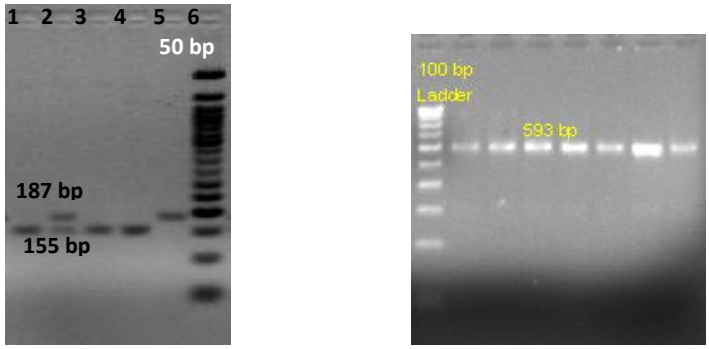
Data are shown as mean ± SD wherever applicable. a is comparison between HC and DWR groups, b is comparison between HC and DR groups, and c is comparison between DWR and DR. * $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$, ^{NS} P value non significant. BMI, Body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure.



(a) (b) (c) (d) (e)

[**Figure 1:** RFLP band patterns of (a) rs699947 (-2578 C / A), (b) rs833069 (3596A/G), (c) rs2146323 (6112 C/A), (d) rs3025021 (10180 T/C) and (e) rs3025039 (13553 C/T) SNPs of VEGF gene on 2.8% agarose gel electrophoresis]

Figure 1 is showing the possible RFLP band patterns of rs699947 (-2578 C/A), rs833069 (3596 A/G), rs2146323 (6112 C/A), rs3025021 (10180 T/C) and rs3025039 (13553 C/T) SNPs of VEGF gene and genotyping for all samples was done using band pattern provided in table 3. The genotypes of SNP-19 is shown in figure 2, where genotyping was done as described in genotyping of SNPs above. Figure 3 shows genotyping for SNP-44 and SNP-43.



(a) (b)

[**Figure 2:** Genotypes on 2.8% agarose gel, where, (a) for SNP-19 of CAPN 10, (b) SNP-43& SNP-44]

We analysed the Genotypic and allelic frequencies of SNPs in association with T2D or DR using two way Fisher exact's test. The analysis of all the SNPs is discussed below.

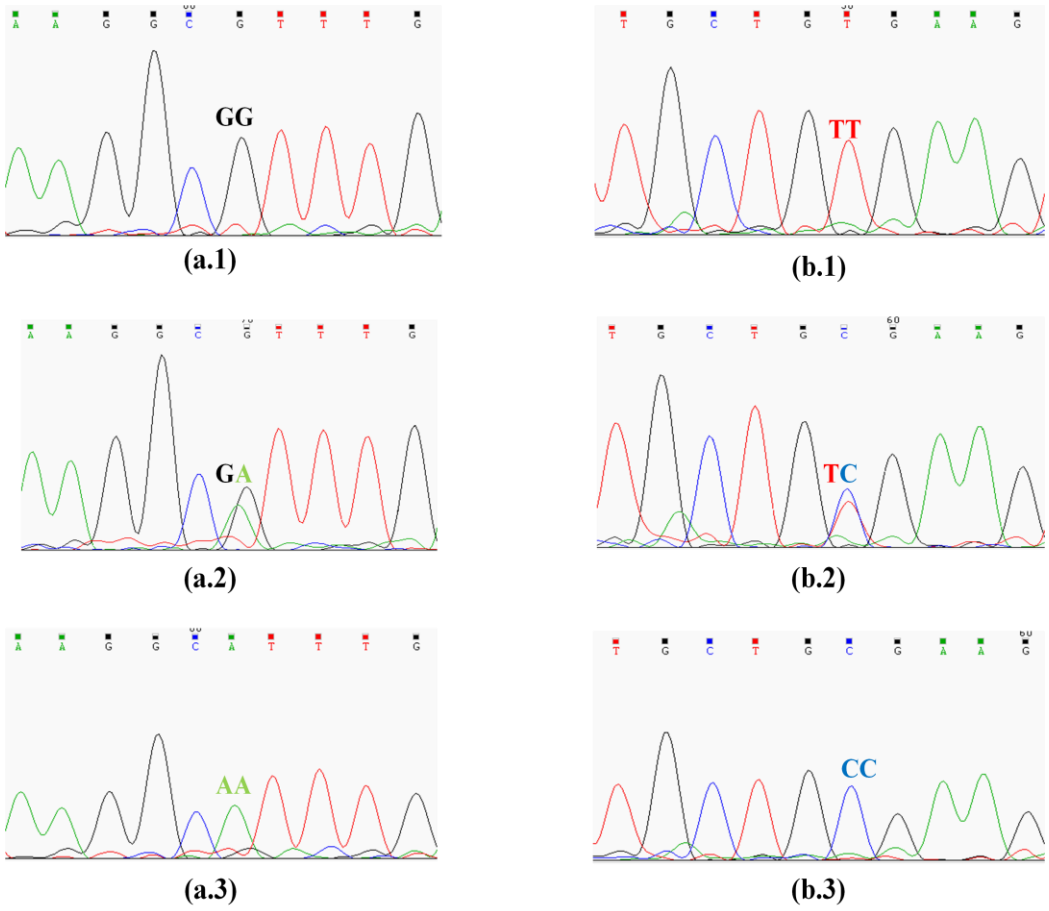


Figure 3: Electrogram of the sequences for SNP-43 and SNP-44. [a] SNP-43 showing its genotypes (a.1) GG (a.2) GA (a.3) AA [b] SNP-44 showing its genotypes (b.1) TT (b.2) TC (b.3) CC

Evaluation of rs699947 (-2578 C>A), rs833061 (-1498 T>C), rs13207351 (-1190 G>A), rs2010963 (-634 C>G), and rs3025039 (13553 C>T) of VEGFA gene

We genotyped common polymorphisms located in the promoter and UTR regions of VEGFA gene. All the SNPs genotyped were in Hardy Weinberg Equilibrium. rs699947 is one of the most frequently studied SNP in association with DR in various populations like Chinese, Japanese, Australian etc (Lu et al, 2013). We observed minor homozygous (AA) genotypic frequency 9.2% and 8% higher among patients suffering from the diabetic retinopathy in comparison to control and DWR individuals, respectively. Likewise, allelic distribution showed 9.1% and 6% increase in the frequency of the A allele in DR group when compared to control and DWR group. In spite of the difference in the genotypic and allelic distribution, Fisher's exact test showed a poor

association of the genotype as well as allele distribution with DR (For AA genotype p = 0.066 and 0.084; for A allele p = 0.057 and 0.177 compared to control and DR). We believe that though rs699947 was not significantly associated with DR in the studied population may be due to low sample size, the recessive model of the SNP may have a minor role in the genetic predisposition to DR.

We genotyped rs833061 (-1498 T>C), rs13207351 (-1190 G>A) and rs2010963 (-634 C>G) using next generation sequencing, where, we studied DWR and DR individuals. Though the genotypic distribution of the SNPs was different in the two study groups, it did not show statistically significant correlation of the SNPs with DR.

The other targeted polymorphism rs3025039 of VEGFA gene was located in the 3' UTR region. We found the heterozygous genotype more frequently among diabetic subjects as compared to control subjects (12.7% in DWR group and 15.5% in DR group vs. 7.5% in control group). Interestingly, none of the individuals was harboring the homozygous minor genotype. There was no difference in the genotypic as well as allelic frequencies between DWR and DR patients. Hence, the present study did not found the SNP to be associated with DR in the targeted population.

Evaluation of the intronic SNPs of VEGFA gene

The HWE for rs833069, rs2146323, and rs3025021 was determined in all study groups; which significantly deviated for rs2146323 polymorphism in the control group. The recessive model of rs2146323 polymorphism was significantly found to be associated with DR when compared to control and DWR groups (p = 0.0005, OR 16.06, 95% CI 2.12 – 121.33 and p = 0.044, OR 2.57, 95% CI 1.06 – 6.25, respectively). We observed that out of 22 DR patients harboring AA genotype, correspondingly 14 (63.6%) and 5 (22.7%) patients were diagnosed with PDR and severe nonproliferative diabetic retinopathy (NPDR), indicating its association more precisely with the severity of DR (p = 0.0001). However, allele distribution of the SNP showed correlation with DR group only when compared to control group (p = 0.023). Moreover, the frequency of AA genotype of rs2146323 was non-significantly higher among DWR patients in comparison with controls (p = 0.073).

The genotype and allele distributions for the other two SNPs, rs833069 and rs3025021 did not vary significantly among the studied groups. However, the frequency of TT genotype was slightly increased among DR patients in comparison with control and DWR subjects ($p = 0.094$ and $p = 0.176$, respectively). Therefore, the current study suggested that rs2146323 polymorphism imparted risk to develop DR, while rs833069 and rs3025021 were not associated with DR in the targeted western part of India. We studied haplotype of the three intronic SNPs, where AAC was increasing significant risk to develop PDR and ACC was indicating the protection against developing PDR.

We included SNP-43, SNP-44, and SNP-19 of CAPN 10 gene in the study and evaluated them for predisposition to T2D and DR. We suggested that DD genotype of SNP-19 was slightly associated with T2D and DR. However, genotypic and allelic distribution of other two SNPs did not show any relation with T2D or DR. The linkage disequilibrium plot (LD) indicated SNP-43 and SNP-44 in a strong linkage. The haplotypes observed were analysed for their association with the disease. We found ATD as a risk haplotype for PDR. SNP – 44 and SNP – 19 showed an association with Body mass Index and glycated haemoglobin, correspondingly.

T2D and DR being a complex disease involves multiple genetic and environmental determinants. For the genetic studies of complex human disease, it has been recognized that the interplay among multiple genetic variants is driving disease phenotype rather than single or a few SNPs. Since, the study included comparatively small sample size, the validation of the suggested SNPs in larger population may increase the significance of them in association with DR. The strengths of the project were including standard diagnosis criteria for the cases by ADA and ETDRS guideline, restricting the study to well-defined Western Indian ethnicity, and consideration of confounders for the risk assessment of the disease.

References:

Evans J C, Frayling T M, Cassell P G, Saker P J, Hitman G A, Walker M, Levy J C, O'Rahilly S, Subba Rao P V, Bennett A J, Jones E C, Menzel S, Prestwich P, Simecek N, Wishart M,6 Ranjit Dhillon, Chris Fletcher, Ann Millward, Andrew Demaine, Terence Wilkin, Yukio Horikawa, Nancy J. Cox, Graeme I. Bell, Sian Ellard, Mark I. McCarthy and Andrew T.

Hattersley. Studies of Association between the Gene for Calpain-10 and Type 2 Diabetes Mellitus in the United Kingdom. Am. J. Hum. Genet., 69:544–552, 2001.

Laird N M, Lange C. Family-based designs in the age of large scale gene association studies. Nature Review Genetics, 7: 385-394; 2006.

Maeda S. Genome-wide search for susceptibility gene to diabetic nephropathy by gene based SNP. Diabetes research and clinical practice, 66s: s45-s47; 2004.

Ray D, Mishra M, Ralph S, Read I, Davies R and Brenchley P.Association of the VEGF Gene with Proliferative Diabetic Retinopathy But Not Proteinuria in Diabetes. Diabetes, 53: 861-864; 2004.

Schlingemann RO, van Hinsbergh VW. Role of vascular permeability factor/vascular endothelial growth factor in eye disease.Br JOphthalmol.81:501–512; 1997.

Suganthalakshmi B, Anand R, Kim R, Mahalakshmi R, Karthikprakash S, Namperumalsamy P, Sundaresan P. Association of *VEGF* and *eNOS* gene polymorphisms in type 2 diabetic retinopathy. Molecular Vision, 12: 336-41; 2006.

Turner MD, Cassell PG, Hitman GA. Calpain-10: from genome search to function, Diab. Metab. Res Rev. 21:505–514; 2005.

Yang et al. Polymorphisms in the vascular endothelial growth factor gene and the risk of diabetic retinopathy in Chinese patients with type 2 diabetes.Molecular Vision 17:3088-3096; 2011.

Zhou YP, Sreenan S, Pan CY, Currie KPM, Bindokas VP, Horikawa Y, et al. A 48-hour exposure of pancreatic islets to Calpain inhibitors impairs mitochondrial fuel metabolism and the exocytosis of insulin, Metabolism 52:528–534; 2003.

iii. Has the progress been according to original plan of work and towards achieving the objective. If not, state reasons: Yes

iv. Please indicate the difficulties, if any, experienced in implementing the project: No

v. If project has not been completed, please indicate the approximate time by which it is likely to be completed. A summary of the work done for the period (Annual basis) may please be sent to the Commission on a separate sheet: The project is completed in the given tenure of the project.

vi. If the project has been completed, please enclose a summary of the findings of the study. One bound copy of the final report of work done may also be sent to University Grants Commission:

Summary of the findings of the study is attached in Annexure IX. One bound copy of the final report of work done is sent to University Grants Commission

vii. Any other information which would help in evaluation of work done on the project. At the completion of the project, the first report should indicate the output, such as

(a) Manpower trained: Project fellow appointed and trained

(b) Ph. D. awarded: Project fellow registered for Ph.D. in Biochemistry in Sardar Patel University (Registration No. 189) (Synopsis Submitted)

(c) Publication of results:

1. Dhara Nareshkumar Jajal and Kiran Kalia. Vascular Endothelial Growth Factor-A (VEGFA) Gene Polymorphisms and Genetic Predisposition of Retinopathy in Type 2 Diabetes Patients of India. International Journal of Advanced Biotechnology and Research, 8(1), 2017, pp209-220.

2. Dhara Nareshkumar Jajal and Kiran Kalia. Investigating common CAPN10 polymorphisms for the susceptibility of Type 2 Diabetes and Diabetic Retinopathy – Manuscript ready to communicate

(d) Other impact, if any:

- Project fellow is going to present a poster entitled "Genetic Variations in Coding Region of Vascular Endothelial Growth Factor (VEGF) and Risk of Diabetic Retinopathy: A Primary Case-control Study" in International Conference "ARVO annual meeting" going to be held at Baltimore, USA from 7 May to 11 May 2017
- Project fellow is going to present a poster entitled "Impact of VEGF Gene Polymorphisms on Progression of Diabetic Retinopathy in an Indian Population" in International Conference "Experimental Biology" going to be held at Chicago, USA from 22 April to 26 April, 2017 and she awarded the Graduate Travel award for the same.
- Project fellow presented poster entitled "Role of VEGFA Gene Polymorphisms in Susceptibility to Diabetic Retinopathy" at 5th International conference on "NextGen Genomics, Biology, Bioinformatics and Technologies (*NGBT*)" held from Oct 3rd-5th, in Cochin, India, organized by SciGenom Research Foundation.
  Awarded full travel scholarship to present at the conference.
- Project fellow participated in workshop on "Advanced Techniques in Genomics of Type 2 Diabetes" Conducted by Madras Diabetes Research Foundation & University of Minnesota, U.S.A held at Madras Diabetes Research Foundation, Chennai from January 21st -24th, 2014.
- Project fellow has presented a poster on "Association of Single Nucleotide Polymorphisms in Superoxide Dismutase with Onset of Type 2 Diabetes and Diabetic Nephropathy" and won 3rd prize for the same at two days national conference organized by Nirma University, Ahmedabad on "Diabetes and Its Complications" held on 6-7 September, 2013.

PRINCIPAL INVESTIGATOR

SARDAR PATEL UNIVERSITY

12.4.17
Registrar
Sardar Patel University
Vallabh Vidyanagar.

REGISTRAR

Annexure - IX

BRD School of Bioscience
Sardar Patel University
Vallabh Vidyanagar – 388 120.

SUBMISSION OF INFORMATION AT THE TIME OF SENDING THE FINAL REPORT OF THE WORK DONE ON THE PROJECT

1. TITLE OF THE PROJECT:
"Association of Single Nucleotide Polymorphisms with Type 2 Diabetes and Diabetic Retinopathy in Western Indian Population"

2. NAME AND ADDRESS OF THE PRINCIPAL INVESTIGATOR
Dr. Kiran Kalia
BRD School of Bioscience
Sardar Patel University
Vallabh Vidyanagar – 388 120

3. NAME AND ADDRESS OF THE INSTITUTION
Dr. Kiran Kalia
BRD School of Bioscience
Sardar Patel University
Vallabh Vidyanagar – 388 120

4. UGC APPROVAL LETTER NO. AND DATE: F.-42-637/2013(SR) dated 22/03/2013

5. DATE OF IMPLEMENTATION: 01/ 04/ 2013

6. TENURE OF THE PROJECT: From 01/ 04/ 2013 to 31/ 03/ 2017

7. TOTAL GRANT ALLOCATED: **13, 75, 800/-**

8. TOTAL GRANT RECEIVED: **12, 21, 800/-**

9. FINAL EXPENDITURE: **13, 65, 799/-**

10. TITLE OF THE PROJECT: "Association of Single Nucleotide Polymorphisms with Type 2 Diabetes and Diabetic Retinopathy in Western Indian Population"

11. OBJECTIVES OF THE PROJECT:

The objective of the project was to study the association of common SNPs in CAPN10 and VEGF genes with type 2 diabetes and diabetic retinopathy in the western part of India.

Following SNPs were studied in VEGF gene in association with type 2 diabetes and diabetic retinopathy in western part of India.

- rs699947 (-2578 C>A), rs833061 (-1498 T>C), rs13207351 (-1190 G>A) of promoter region; rs2010963 (-634 C>G) of 5' UTR; rs833069 (3596 A>G), rs2146323 (6112 C>A) of intron 2; rs3025021 (10180 T>C) of intron 6; rs3025039 (13553 C>T) of 3' UTR.

- To study association of SNP19 (rs3842570), SNP43 (rs3792267) and SNP63 (rs5030952) of CAPN10 gene with type 2 diabetes and diabetic retinopathy in western part of Indian population.

12. WHETHER OBJECTIVES WERE ACHIEVED: Yes

We have successfully completed the objectives for which details are given in the Annexure VIII

13. ACHIEVEMENTS FROM THE PROJECT:

We successfully genotyped single nucleotide polymorphisms in type 2 diabetes, diabetic retinopathy and healthy individuals. We obtained the association of the two SNPs with diabetic retinopathy that give insights to other researchers to further validated them in large cohort to use them as markers for early detection of the disease.

The project has trained project fellow in the field of genotyping and sequencing of the DNA material, who is going to complete PhD in coming months.

14. SUMMARY OF THE FINDINGS

We studied common polymorphisms in Calpain 10(CAPN10) and Vascular Endothelial Growth Factor (VEGF) genes to find out their association with type 2 diabetes (T2D) and diabetic retinopathy (DR). Out of all targeted intronic SNPs in VEGF gene, rs2146323 was significantly associated with diabetic retinopathy and prolifertive diabetic retinopathy (PDR) in comparison with healthy group and diabetic group without any complication ($p < 0.05$). The minor homozygous genotype of the SNP was increasing almost 1.2 and 2.1 fold risk to develop DR and PDR, respectively. The other two intronic SNPs of VEGF, rs833069 and rs3025021were not differently distributed among the study groups. However, TT genotype of rs3025021 was slightly associated with PDR. We also studied haplotype combination of the three intronic SNPs, where

we observed AAC haplotype significantly imparting risk to severe DR i.e. PDR and ACC is a protective haplotype for PDR. Among all other promoter and UTR region SNPs, only rs699947 has shown minor role in developing DR ($p = 0.066$) when compare to healthy individuals and not with DWR individuals. The association may become more significant on inclusion of the large sample size. On the other hand, all the other SNPs did not show a significant correlation with T2D or DR.

We included SNP-43, SNP-44, and SNP-19 of CAPN 10 gene in the study and evaluated them for predisposition to T2D and DR. We suggested that DD genotype of SNP-19 was slightly associated with T2D and DR. However, genotypic and allelic distribution of other two SNPs did not show any relation with T2D or DR. The linkage disequilibrium plot (LD) indicated SNP-43 and SNP-44 in a strong linkage. The haplotypes observed were analysed for their association with the disease. We found ATD as a risk haplotype for PDR. SNP – 44 and SNP – 19 showed an association with Bodymass Index and glycated haemoglobin, correspondingly.

In summary, we suggest that few SNPs as described above may play crucial role in severe diabetic retinopathy in the current population, while they are less likely to be associated with type 2 diabetes.

15. CONTRIBUTION TO THE SOCIETY:

Since, available treatment and/ or interventional strategies are not sufficient to combat with the rising prevalence of type 2 diabetes (T2D) diabetic retinopathy (DR); there is a strong need for early diagnosis and/or detection of the people at high risk of developing T2D and DR. It has been established that genetic factors confer risk to develop T2D and DR. The genetic association studies require trans-racial approach and need to be validated in our Indian population. We studied genetic variations in CAPN 10 and VEGF genes in association with T2D and DR in the Western part of India, which has been not studied till date. We found that rs2146323 is strongly associated with DR and not with T2D. Moreover, rs699947 was slightly increasing risk for DR. Both the SNPs can be studied in a large population further. The study would, in turn, assist in identifying individuals at high risk of DR in the Western Indian population, which would help in the early disease management and delay the onset or severity of DR by appropriate preventive treatments or interventional strategies.

16. WHETHER ANY PH.D. ENROLLED/PRODUCED OUT OF THE PROJECT: Yes

Project fellow, Dhara Jajal has registered for Ph.D. in Biochemistry in Sardar Patel University (Registration No. 189) (Synopsis Submitted- 24th October, 2016. Thesis likely to be submitting by May, 2017)

17. NO. OF PUBLICATIONS OUT OF THE PROJECT

1. Dhara Nareshkumar Jajal and Kiran Kalia. Vascular Endothelial Growth Factor-A (VEGFA) Gene Polymorphisms and Genetic Predisposition of Retinopathy in Type 2 Diabetes Patients of India. International Journal of Advanced Biotechnology and Research, 8(1), 2017, pp209-220.

2. Dhara Nareshkumar Jajal and Kiran Kalia. Investigating common CAPN10 polymorphisms for the susceptibility of Type 2 Diabetes and Diabetic Retinopathy – Manuscript ready to communicate

3. Sejal Shah, **Dhara Jajal**, Girish Mishra, Kiran Kalia (2016) Genetic profile of the PTEN Gene in Indian OSCC Primary Tumors. Journal of Oral pathology and Medicine. DOI: 10.1111/jop.12468

Manuscript under Preparation: 1
(PLEASE ATTACH)

PRINCIPAL INVESTIGATOR

Dr. (Mrs.) Kiran Kalia
Principal Investig.

SARDAR PATEL UNIVERSITY

RHRajput 12.4.17
Registrar
Sardar Patel University
Vallabh Vidyanagar.

REGISTRAR/PRINCIPAL

International Journal of Advanced Biotechnology and Research (IJBR)
ISSN 0976-2612, Online ISSN 2278–599X,
Vol-8, Issue-1, 2017, 209-220
http://www.bipublication.com

Research Article

Vascular Endothelial Growth Factor-A (*VEGFA*) Gene Polymorphisms and Genetic Predisposition of Retinopathy in Type 2 Diabetes Patients of India

Dhara Nareshkumar Jajal[1] and Kiran Kalia[1,2]

[1]Lab # 103B, B. R. D. School of Biosciences, Sardar Patel University,
Vallabh Vidyanagar – 388 120, Gujarat, India
[2]National Institute of Pharmaceutical Education & Research – Ahmedabad,
Nr. Palaj Village, Gandhinagar - 382355, Gujarat, India
[*]Corresponding author: Kiran Kalia, Email: kirankalia@gmail.com, director@niperahm.ac.in,
Tel: +91-9714618573, +91-9824335881; Fax: +91-79- 27450449

ABSTRACT:

Purpose: Vascular Endothelial Growth Factor - A (VEGFA) promotes angiogenesis and its role in the pathology of diabetic retinopathy (DR) is well documented. Although the polymorphisms in VEGFA gene have been shown to increase the risk of DR development and progression in various ethnicities, few studies have been carried out targeting the intronic SNPs. Therefore, the main purpose of present study was to assess the genetic predisposition of DR and proliferative DR (PDR) attributed by three intronic polymorphisms of VEGFA gene among type 2 diabetes (T2D) patients.

Method: We enrolled total 351 unrelated individuals [93 healthy controls (HC), 110 T2D patients without retinopathy (DWR) and 148 T2D patients with retinopathy (DR)] from the western region of India. Genotyping of rs833069, rs2146323, and rs3025021 SNPs was performed by PCR-RFLP.

Results: The AA genotype in a co-dominant model and minor allele (A) of rs2146323 was significantly high in PDR patients when compared to DWR patients ($p = 0.003$ and $p = 0.010$, respectively). However, the SNP was not significantly associated with DR when compared to HC or DWR individuals on applying multivariate logistic regression ($p = 0.142$ and $p = 0.045$, correspondingly). We did not observe significant variation in the distribution of rs833069 and rs3025021 polymorphisms among the study groups. Our data suggested rs833069 and rs2146323 SNPs were in linkage disequilibrium ($D'=0.947$), and ACC of the observed haplotypes showed a significant inverse association with PDR ($p = 0.001$).

Conclusion: Our study suggested that minor homozygous genotype of rs2146323 conferred two-fold risk to develop PDR in the targeted Indian ethnicity. Further studies in larger population would help in confirming the association substantially.

Keywords: VEGFA gene, Diabetic retinopathy, Intronic SNPs, Indian population

[I] INTRODUCTION

Diabetic retinopathy (DR) - a microvascular complication of diabetes, is a well-recognized consequence of long-standing and poorly controlled hyperglycemia. It is estimated that DR affects from 12 to 30% of the diabetic population in India [1]. DR is chiefly characterized by retinal microaneurysms, vascular exudations of proteins in retina or macula, and subsequent neovascular proliferation accompanied by vitreous hemorrhage [2]. The precise molecular mechanism of DR is poorly understood due to its multifaceted pathogenesis. Moreover, the conventional risk

factors like prolonged hyperglycemia and oxidative stress do not entirely explain the etiology of DR [3,4].

Previous studies have evidently shown the ethnicity specific high prevalence and heritability of DR, including an Indian population [5-8]. Thus, genetic factors are well established in the predisposition of DR, and their identification is an area of substantial research for developing screening algorithms for early detection of DR. Several studies have reported potential genes associated with DR prevalence that evidently includes vascular endothelial growth factor - A (VEGFA).

It is an endothelial cell specific chemokine that mediates angiogenesis and has been known to play a pivotal role in the pathogenesis of DR. VEGFA expression is stimulated by oxidative stress produced in diabetic retina. As a result, markedly elevated levels of VEGFA have been reported in ocular fluid of patients with DR [9-11].

During the DR progression, VEGFA mediates breakdown of blood-retinal barrier and ischemia-induced neovascularization that is hyperpermeable, the condition known as proliferative diabetic retinopathy (PDR) [10,11]. It can eventually lead to retinal detachment and ultimately to blindness if left untreated. However, therapeutic administration of VEGFA antagonists has demonstrated reduced retinal permeability and neovascularization [12,13].

The human VEGFA gene is mapped on chromosome region, 6p21.3, which spans to approximately 18kb including promoter region and it is highly polymorphic.

The VEGFA consists of eight exons, and its alternative splicing refers to the two VEGFA protein families, namely, pro-angiogenic and anti-angiogenic [14]. So far, the most investigations have analyzed polymorphisms in the 5′ untranslated region and promoter of the VEGFA for finding their influence on the susceptibility of DR and/or PDR.

Nevertheless, fewer studies have taken the intronic variations of VEGFA into consideration for the same. The previous studies targeting intronic SNPs of VEGFA gene that may increase the DR occurrence among type 1 and type 2 diabetic (T2D) patients have shown widely conflicting results [15-21].

Moreover, genetic association studies require trans-racial approach since ethnicity influences the allele frequencies of SNPs and their linkage disequilibria.

Therefore, we aimed to evaluate the common intronic SNPs of VEGFA gene (rs833069, and rs2146323 of intron 2, and rs3025021 of intron 6) for predisposing our distinct Indian T2D population to DR and PDR;

which were not investigated earlier in the Indian population as per our knowledge. The studied SNPs are illustrated on VEGFA gene map in figure 1.

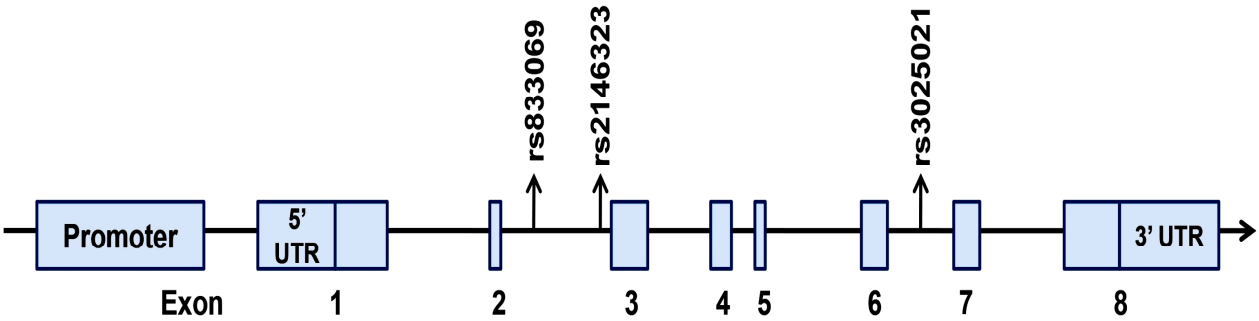


Figure: 1. *VEGFA* gene map showing the locations of the three SNPs analyzed in the study

[II] MATERIALS AND METHODS

2.1. Participants

The present study was approved by Human Research Ethics Committee of Pramukh Swami Medical College and Hospital, Karamsad, Gujarat. The study was conducted as per the principles embodied in the Declaration of Helsinki. We made all participants acquainted with the research and obtained informed consents before sample collection. In our cross-sectional study, we incorporated 258 T2D patients who had attended Pramukh Swami Medical College and Hospital, Karamsad, Gujarat, from March 2013 to May 2015. The study comprised of total four groups:

1. Healthy controls (HC, n = 93)
2. T2D patients without retinopathy (DWR, n = 110)
3. T2D patients with retinopathy (DR, n = 148)
4. T2D patients with proliferative retinopathy – a subgroup of DR (PDR, n = 62)

The treating physician diagnosed the patients with T2D as per the American Diabetes Association (ADA) guidelines. All the patients and controls were undergone visual acuity and fundus examination through dilated pupils. An expert ophthalmologist diagnosed them for DR grading according to Diabetic Retinopathy Disease Severity Scale based on Early Treatment Diabetic Retinopathy Study (ETDRS) [22].

The HC group was characterized by normal blood pressure, normal fundus test, the absence of parental diabetes history and any other diseased condition. They were volunteers, blood donors, or relatives to the patients that visited the hospital. We interviewed all subjects through a structured questionnaire, and their clinical characteristics were noted.

All subjects belonged to the western part of India, more precisely Gujarat, and hence were from the same geographical area and culture. The patients with malignancy, inflammatory diseases, other genetic diseases, amputations, end stage renal disease, history of cardiac stroke or bypass surgery, paralysis were excluded from the study.

2.2. Biochemical Analysis

Fasting blood samples were collected in EDTA-coated Vacutainers (BD Biosciences) and transported to the laboratory at 4 °C. The fasting plasma glucose (FPG) and serum creatinine levels were measured by GOD-POD and Jaffe's reaction based kits, respectively. The glycated hemoglobin (GHb) was estimated by a conventional thiobarbituric acid method.

2.3. Genotyping of the SNPs

The DNA extraction was carried out using QIAamp® DNA Blood kit (Qiagen) by following the manufacturer's protocol. The DNA samples with absorption at 260 nm/ 280 nm $\geq$ 1.8 were used for genotyping of the SNPs. We performed PCR-RFLP to genotype rs833069 T>C, rs2146323 C>A, and rs3025021 T>C SNPs in all participants. The specific PCR primers were designed by the Primer-Blast tool of NCBI. All samples were amplified in a 25 ul reaction mixture using Applied Biosystems® Veriti® thermal cycler.

Each reaction contained 12.5 ul Taq 2x master mix (New England BioLabs), 400-450 nM forward and reverse primers (Eurofins), 40 ng DNA template, and H2O to make up the volume. Table 1 is representing the primers used in the study, their respective Tm, and product length. The PCR cycling conditions were: denaturing at 95ºC for 1 min, 1 cycle; denaturing at 94ºC for 30 s, annealing at the pertinent Tm for 20 s, extension at 68ºC for 30 s, 30 cycles and a final extension at 68ºC for 5 min. The 2.5% agarose gel was run to verify the specific PCR products visualized by ethidium bromide staining.

The restriction enzymes were selected by an online application, NEBcutter. The selected restriction enzymes and their corresponding fragment pattern to genotype the SNPs are summarized in Table 1.

SNP	Primer	Tm	Product Size	RE	Fragment Pattern
rs833069	F: 5'GTTCACAGCACCCGAACATA3' R: 5'GAACAGCGGAGAGTCCTCAC3'	58 ºC	358 bp	BseRI	C allele: 358 bp T allele: 274 bp & 84 bp
rs2146323	F: 5'GTCTCGATTGGATGGCAGTA3' R: 5'CCCATACTCAGACTGTCCTCT3'	57 ºC	384 bp	MluCI	C allele: 384 bp A allele: 224 bp & 160 bp
rs3025021	F: 5'TTCCACCAAGGTGGGCTAAA3' R: 5'CTGCTCACCCAACTGGTTTC3'	60 ºC	352 bp	NciI	T allele: 333 bp & 19 bp C allele: 260 bp, 73 bp & 19 bp

Table 1: Primers, their corresponding Tm and product size, and restriction enzymes with their fragment pattern used to genotype the SNPs

The restriction digestion was done in a 25 ul reaction containing 1x Cutsmart buffer (New England BioLabs), 10 ul of PCR product and 2 U of specific restriction enzyme (New England BioLabs). The reactions for all SNPs were incubated at 37ºC for 6-8 h for complete restriction digestion.

The digested PCR products were run on a 3% agarose gel, and the genotyping was done according to the visualized fragments.

2.4. Statistical Analysis

The study used the R statistical package to perform the statistical analysis of the data. Baseline demographic and biochemical characteristics were compared between study groups by Kruskal-Wallis test and one-way ANOVA for non-parametric and parametric continuous variables, respectively.

The assessment of categorical covariates was done by two-tailed Fisher's exact test. The chi-square test was performed to examine the Hardy–Weinberg equilibrium (HWE) at the individual polymorphic locus.

The p-value less than 0.05 was considered statistically significant. Univariate and multivariate logistic regression analyses were carried out to analyze the distribution of SNPs among the study groups. Boneferroni correction was applied for multiple testing for which statistically significant cutoff p-value was less than 0.016. Additionally, odds ratios (OR) with 95% confidence intervals (CI) were estimated to identify the risk of DR and PDR. Linkage disequilibrium (LD) between SNPs and association of observed haplotypes were determined by SHEsis software [23,24].

[III] RESULTS

We studied total 351 individuals, out of which 110 T2D patients did not show DR characteristics that were designated as DWR group. On the other hand, 148 patients were diagnosed for DR, out of which 62 patients showed characteristics of PDR at least in one eye. Moreover, more than half (65%) of the DR patients were diagnosed with grade 4 or above on ETDRS severity scale.

3.1. Demographic, Clinical and Biochemical Findings of the Study Groups

The demographic, clinical and biochemical details of the studied groups are given in Table 2. The DR patients were significantly older in age than HC and DWR subjects, whereas, when the age at the onset of T2D was considered, it did not show the variation among HC, DWR and DR groups (mean age 48.3, 47.8, and 49.8, correspondingly). Systolic blood pressure was significantly higher in T2D patients than in HC, whereas, only DR group presented significant high diastolic blood pressure than the healthy HC group. We observed that 31.8% and 61.5% of the DR individuals were correspondingly having diabetes for ≤ 5 years and ≤ 10 years, indicating early onset of retinopathy in the studied population. Conversely, the other confounders like the presence of hypertension, duration of diabetes, family history of diabetes and habits were non-significantly different between both the groups of T2D. As shown in Table 2, all the biochemical parameters were found to be significantly elevated in DR patients when compared to DWR and HC participants. Further, DWR subjects showed apparent increased levels of FPG and GHb than HC individuals ($P < 0.0001$).

Table 2: Clinical and biochemical characteristics of the study groups

Parameters	HC	DWR	DR	P value
Number	93	110	148	
Age (years)	48.3 ± 11.5	55.3 ± 9.0	59.1 ± 7.4	a*** b*** c**
Gender [n (%)]				
Male	53 (57.0)	66 (60.0)	88 (59.5)	$(a\ b\ c)^{NS}$
Female	40 (43.0)	44 (40.0)	60 (40.5)	
BMI (kg/m^2)	24.3 ± 3.9	26.4 ± 4.0	24.9 ± 4.0	a** b^{NS} c*
Hypertensive Patients [n (%)]		59 (53.6)	95 (64.2)	C^{NS}
SBP (mm hg)	122.0 ± 7.4	131.9 ± 16.4	132.7 ± 14.1	a*** b*** c^{NS}
DBP (mm hg)	79.5 ± 5.6	80.9 ± 9.2	82.6 ± 9.2	a^{NS} b* c^{NS}
Duration of diabetes (years)	-	7.5 ± 3.3	8.9 ± 5.4	C^{NS}
Unknown	-	0	7	
Hypoglycaemic agents [n (%)]				
Oral Hypoglycaemic agents	-	101 (91.8)	115 (77.7)	C*
Insulin	-	3 (2.7)	13 (8.8)	
Oral + Insulin	-	4 (3.7)	14 (9.5)	
No medications	-	2 (1.8)	6 (4.0)	
Family History of Diabetes [n (%)]		51 (49.0)	66 (46.2)	
Mother	-	29	29	C^{NS}
Father	-	25	20	
Siblings	-	15	35	
Unknown	-	6	5	
Habits [n (%)]		43 (39.1)	54 (36.5)	
Tobacco chewing/ sniffing	-	26/7	29/2	C^{NS}
Smoking	-	13	17	
Occasional/ former Habituate	-	3/0	8/2	
Fasting Plasma Glucose (mg/dl)	84.5 ± 7.2	130.2 ± 20.6	138.9 ± 26.4	a*** b*** c*
Glycated Hemoglobin (%)	6.31 ± 0.68	8.99 ± 0.71	9.57 ± 1.04	a*** b*** c***
Serum Creatinine (mg/dl)	0.99 ± 0.26	1.01 ± 0.28	1.12 ± 0.31	a^{NS} b** c*

Data are shown as mean ± SD wherever applicable. a is comparison between HC and DWR groups, b is comparison between HC and DR groups, and c is comparison between DWR and DR. * $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$, ^{NS} P value non significant. BMI, Body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure.

Table 3: Genotypic and Allelic distributions of VEGF gene polymorphisms among studied groups

Genotype/ Allele	HC [n (%)]	DWR [n (%)]	DR [n (%)]	PDR [n (%)]
rs833069 (3596 T>C)				
TT	53 (57.0)	64 (58.2)	93 (62.8)	36 (58.1)
TC	37 (39.8)	42 (38.2)	49 (33.1)	23 (37.1)
CC	3 (3.2)	4 (3.6)	6 (4.1)	3 (4.8)
T Allele	143 (76.9)	170 (77.3)	235 (79.4)	95 (76.6)
C Allele	43 (23.1)	50 (22.7)	61 (20.6)	29 (23.4)
rs2146323 (6112C>A)				
CC	42 (45.2)	49 (44.5)	57 (38.5)	19 (30.6)
CA	50 (53.7)	54 (49.1)	69 (46.6)	30 (48.4)
AA	1 (1.1)	7 (6.4)	22 (14.9)	13 (21.0)
C Allele	134 (72.0)	152 (69.1)	183 (61.8)	68 (54.8)
A Allele	52 (28.0)	68 (30.9)	113 (38.2)	56 (45.2)
rs3025021 (10180C>T)				
CC	57 (61.3)	54 (49.1)	79 (53.4)	31 (50.0)
CT	31 (33.3)	50 (45.5)	53 (35.8)	21 (33.9)
TT	5 (5.4)	6 (5.4)	16 (10.8)	10 (16.1)
C Allele	145 (78.0)	158 (71.8)	211 (71.3)	83 (66.9)
T Allele	41 (22.0)	62 (28.2)	85 (28.7)	41 (33.1)

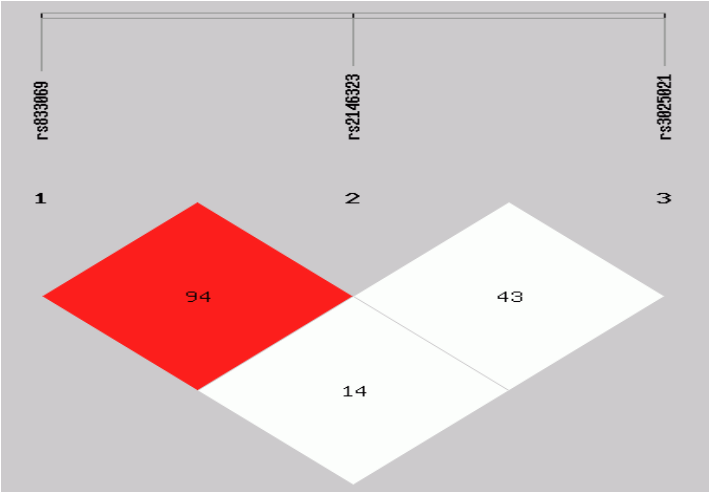


Figure: 2. Linkage disequilibrium (LD) plot of the studied SNPs**:** LD is displayed in the boxes as the pairwise D' value multiplied by 100. The magnitude and significance of the pairwise LD is represented with a red-to-white gradient reflecting higher-to-lower LD values.

3.2. Association of the SNPs with DR

The analyzed SNPs were in Hardy–Weinberg equilibrium in all study groups. Table 3 demonstrates the genotype and allele frequencies of targeted SNPs in the study groups. The univariate analysis of the SNPs in a co-dominant model indicated a significant correlation of AA genotype of rs2146323 with DR when compared to HC (p = 0.0004, OR 2.22, 95% CI 1.44 - 3.43),

but not when compared to DWR (p = 0.033, OR 1.25, 95% CI 1.02 - 1.53) **[Table 4]**.

However, in comparison with DWR patients, PDR patients were having the significant high frequency of AA genotype for univariate as well as multivariate logistic regression analysis (p = 0.002, OR 2.10, 95% CI 1.31 -3.36 and p = 0.003, OR 1.95, 95% CI 1.27 -2.99, respectively). We observed that out of 22 DR patients harboring AA genotype, correspondingly 13 (63.6%) and 6 (22.7%) patients were diagnosed with PDR and severe nonproliferative diabetic retinopathy (NPDR). Moreover, the frequency of AA genotype of the SNP was non-significantly higher among DWR patients in comparison with HC subjects **[Table 4]**. Further, the multivariate analysis for rs2142363 suggested that apart from AA genotype, FBG, serum creatinine, and age were the other confounding factors responsible significantly for developing PDR in the targated population.

As shown in Table 4, the genotype and allele distributions for the other two SNPs, rs833069 and rs3025021 did not vary significantly among the studied groups. Hence, we report a lack of correlation of both the SNPs with DR or PDR in the targeted Indian ethnicity. The LD analysis revealed the strong linkage disequilibrium between rs833069 and rs2142363 having D' value of 0.947 **[Figure 2]**.

The haplotype ACC (rs833069, rs2142363, rs3025021) was inversely associated with PDR group in comparison to DWR group (Haplotype frequency 0.14 vs 0.31, p = 0.001); suggesting ACC as a protective haplotype for severe DR. Conversely, none of the observed haplotypes indicated a significant association with DR.

[IV] DISCUSSION

The present study examined the association of the three intronic SNPs of VEGFA gene with the prevalence of DR and PDR in the Indian subset. One of the frequently studied SNP among them was rs2146323 (C/A) that is located in intron 2 of VEGFA gene. Previously, the SNP was genotyped in Caucasian, Finish, Chinese, and Australian populations for assessing its correlation with DR and/or PDR **[Table 5]**.

In our study, AA genotype of rs2146323 attributed two-fold risk of developing PDR among T2D patients (p = 0.003) which was not observed for DR. Further, the identified risk genotype was significantly associated with the severity of DR (i.e. PDR and severe NPDR patients) (p = 0.0001). On the contrary, the case-control studies in Australian, and Finish populations did not show an association with the same [15, 18].

Opposite to our findings, the C allele of rs2146323 was suggested as a potent risk factor for the development of PDR among type 1 and 2 diabetic Caucasians [17]. In partial affirmation with our results, A allele of rs20146323 was found to be associated with DR in the later study of Yang et al. (2014) (P = 0.004) and not in their earlier study; suggesting sample size play a crucial role in the risk assessment [20, 21].

Table 4: Comparisons of Genotypic and Allelic distribution of VEGF gene polymorphisms between the study groups

SNP	Comparison	HC vs DWR		HC vs DR		DWR vs DR		DWR vs PDR	
		P value	**OR (95% CI)**	**P value**	**OR (95% CI)**	**P value**	**OR (95% CI)**	**P value**	**OR (95% CI)**
	TT vs TC	0.834	0.98 (0.85 -1.14)	0.313	0.87 (0.67-1.13)	0.411	0.95(0.83 -1.08)	0.937	0.99 (0.73 -1.34)
rs833069		0.112*	0.96 (0.91 -1.01)*	0.182*	0.92 (0.82 -1.04)*	0.156*	0.91 (0.80 -1.04)*	0.579*	0.92 (0.70 -1.22)*
(3596 T>C)	TT vs CC	0.901	1.02 (0.70 -1.50)	0.86	1.06 (0.55 -2.05)	0.962	1.01 (0.73-1.38)	0.718	1.15 (0.55-2.41)
		0.351*	0.94 (0.82 -1.08)*	0.965*	1.01 (0.75 -1.35)*	0.402*	0.88 (0.64 -1.19)*	0.905*	0.96 (0.49 -1.87)*
	CC vs CA	0.787	0.98 (0.85 - 1.13)	0.867	1.02 (0.79 -1.33)	0.722	1.02 (0.90 -1.16)	0.313	1.17 (0.86 -1.58)
rs2146323		0.914*	0.99 (0.95 - 1.05)*	0.580*	0.97 (0.86 -1.09)*	0.926*	1.01 (0.89 - 1.14)*	0.468*	1.11 (0.84 -1.45)*
(6112C>A)	CC vs AA	0.068	1.40 (0.98 - 2.01)	0.0004	2.22 (1.44 -3.43)	0.033	1.25 (1.02 -1.53)	**0.002**	**2.10 (1.31 -3.36)**
		0.110*	1.11 (0.98 - 1.27)*	0.142*	1.17 (0.95 -1.44)*	0.045*	1.23 (1.01 -1.50)*	**0.003***	**1.95 (1.27 -2.99)***
	CC vs CT	0.074	1.14 (0.99 - 1.31)	0.436	1.11 (0.85 -1.46)	0.222	0.92 (0.81 -1.05)	0.369	0.87 (0.65 - 1.18)
rs3025021		0.359*	0.98 (0.93 - 1.03)*	0.398*	1.05 (0.93 -1.19)*	0.382*	0.95 (0.83 - 1.07)*	0.496*	0.91 (0.69 -1.20)*
(10180C>T)	CC vs TT	0.708	1.06 (0.78 - 1.44)	0.123	1.44 (0.91 -2.28)	0.242	1.14 (0.91 -1.43)	0.046	1.68 (1.013 -2.80)
		0.403*	0.85 (0.64 - 1.12)*	0.676*	1.04 (0.85 -1.28)*	0.551*	1.07 (0.85 -1.34)*	0.571*	1.15 (0.71 -1.88)*

*indicates adjusted P value or adjusted odds ratio with 95% confidence interval where both represent data after adjustment for covariates including age, diabetes duration, family history of diabetes, hypertension, glycated hemoglobin (GHb), fasting plasma glucose and serum creatinine levels.

Besides cross-sectional studies, longitudinal studies have been carried out in North America and Japan which showed the earliest evidence that A allele of rs2146323 polymorphism influences the progression of DR in type 1 diabetes patients [16, 19]. Both the studies have included rs3025021, where discrepantly Al-Kateb et al. found it a potential risk factor for severe DR. Interestingly, the Asian ethnicities did not identify the role of rs3025021 in DR development so far **[Table 5]**. Similar to the present study, Yang et al. have not found the significant difference in the genotypic and allelic distributions of rs833069 between DWR and DR groups [20, 21]. Though our some results were similar to the Asian ethnicities, the discrepancy was observed in the genotypic and allelic distributions among DWR and DR patients as shown in Table 5.

SNP	Population	Genotype Frequency (homozygous major/ heterozygous/ homozygous minor)		Minor Allele Frequency		Type of Diabetes	Association	Sample size DWR/DR	Reference
		DWR	DR	DWR	DR				
rs833069 (TT/TC/CC)	Chinese	27.3/51.8/20.9	27.1/55/17.8	46.8	45.3	2	DWR vs DR	139/129	[20]
	Chinese	30.5/51.8/20.6	32.6/48.8/18.6	46.5	43	2	DWR vs DR	282/215	[21]
	Present Study	58.2/38.2/3.6	62.8/33.1/4.1	22.7	20.6	2	DWR vs DR, PDR	110/148	
rs2146323 (CC/CA/AA)	Caucasian DCCT/EDIC cohort	-	-	-	35.7	1	DWR vs Severe DR*	1362	[16]
	Caucasians of Northern Europe	42.6/36.1/21.3	42.2/57.8/0	39.4	28.9	1 & 2	DWR vs PDR**	61/45	[17]
	Australian	47/38/15	31/59/11	34.0	40.5	1	DWR vs Blinding DR	93/75	[15]
	Australian	47/43/10	39/50/10	31.5	35.0	2	DWR vs Blinding DR	182/137	[15]
	Japanese	-	55.2/ 37.9/ 6.9	-	25.9	1	DWR vs Severe NPDR*	174	[19]
	Finish	41/45/13	37/48/15	36	39	1 & 2	DWR vs DR	98/131	[18]
	Chinese	63.8/30.4/5.8	52.3/35.9/11.7	21.0	29.7	2	DWR vs DR	168/98	[20]
	Chinese	60.5/33.7/5.8	52.3/33.6/14	22.6	30.8	2	DWR vs DR*	276/214	[21]
	Present Study	44.5/49.1/6.4	38.5/46.6/14.9	30.9	38.2	2	DWR vs PDR**	110/148	
rs3025021 (CC/CT/TT)	Caucasian DCCT/EDIC cohort	-	-	-	32.3	1	DWR vs Severe DR**	1341	[16]
	Japanese	-	75.3/ 22.4/ 2.3	-	13.5	1	DWR vs Mild NPDR, Severe NPDR, PDR	174	[19]
	Australian	39/48/13	39/50/11	37	36	1	DWR vs Blinding DR	94/76	[15]
	Australian	48/38/14	50.4/45.3/4.3	33	37.5	2	DWR vs Blinding DR**	184/139	[15]
	Chinese	66.2/28.7/5.1	78.9/18/3.1	12.1	19.5	2	DWR vs DR	139/129	[20]
	Chinese	67.7/29/3.2	75/22.6/2.4	17.7	13.7	2	DWR vs DR	279/212	[21]
	Present Study	49.1/45.5/5.4	53.4/35.8/10.8	28.2	28.7	2	DWR vs DR, PDR	110/148	

Table 5: Genotype and Allele frequencies of the targeted intronic polymorphisms in the *VEGF* gene among various published studies

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

The rationale behind diverse outcome could be variations in sample sizes, ethnicity, inclusion criteria of cases and controls, demographic factors and study designs. Apart from rs2146323, the intronic polymorphisms of VEGFA gene did not impose a risk to DR or PDR in the current population. There might be role of mutations/ polymorphisms in VEGFA gene or in other genes like transcription factors such as hypoxia-inducible factor 1-alpha, which regulate VEGF expression and not covered in the study [25].

The regulation of VEGFA protein expression has been shown to be influenced by SNPs in the VEGFA gene, mostly in the promoter and 5' UTR regions suggesting their plausible role in DR pathophysiology [26, 27]. However, the exact mechanism by which the intronic SNPs influence DR susceptibility is obscure. The SNPs in the intron region can be located at exonic enhancers or silencers that regulate transcription and splicing of the VEGFA gene, have not been extensively investigated thus far. The modification in splice sites may affect alternative splicing of VEGF165, a predominating isoform in the eye. This may be the reason for the increased ratio of angiogenic and antiangiogenic isoforms observed during DR [28], but no functional study has been carried out suggesting it. The polymorphisms, rs833069, rs2146323, and rs3025021 are correspondingly located 450 bp 3' to exon 2, 111-bp 5'to exon 3 and 530 bp 5' to exon 7 **[Figure 1]**. At present, various in-silico analyses are predominating to understand the possible role of intronic SNPs in the disease development. ESE Finder, a web-based tool, suggested the presence of C allele at rs833069 increases the potentiality of the branch site and inserts a new splicing site for SRF1 (Serine-rich splicing factor) [29, 30]. On the other hand, rs2146323 and rs3025021 are not located within the predicted splicing factor binding sites, making them little less attractive for the functional analysis. However, the minor allele of rs3025021 showed putative insertion in ESE sites for SRF2 and SRF6. It is perhaps more likely that these SNPs serve to highlight an as-yet-unknown variant. The other possibility for the SNPs in predisposing diabetic patients to DR can be their possible high linkage disequilibrium in particular ethnicity with other SNPs that has functional and /or genetic association with DR.

DR being a complex disease involves multiple genetic and environmental determinants. For the genetic studies of complex human disease, it has been recognized that the interplay among multiple genetic variants is driving disease phenotype rather than single or a few SNPs. The limitation of the study was that other polymorphisms of VEGFA gene and genes other than VEGFA were not taken under consideration that would have influenced the DR susceptibility. Moreover, the current cross-sectional study included comparatively small sample size which may limit the power of the outcome. However, the strengths were including standard diagnosis criteria for the cases by ADA and ETDRS guideline, restricting the study to well-defined Indian ethnicity, and consideration of confounders for the risk assessment of the disease.

[V] CONCLUSION

In summary, our data suggested the potential relationship between the recessive genotype and allele of rs2146323 of VEGFA and PDR. However, other two SNPs did not confer risk to develop DR or PDR. Further, ACC haplotype showed a significant protective effect for PDR among T2D patients. There is a need of studies with larger sample size in the current Indian population that would help in revealing the findings in a better way. Eventually, such candidate genetic studies would assist in identifying patients at high risk of DR comparatively early and can be delayed with more frequent retinal monitoring programs.

FINANCIAL DISCLOSURE

The present study was financially supported by the University Grant Commission (UGC), New Delhi, India under "Major Research Project"

Scheme (Sanction Letter No. F.-42-637/2013(SR) dated 22/03/2013).

ACKNOWLEDGEMENT

We are grateful to UGC, New Delhi, India for their financial support for conducting the present research and providing fellowship to Ms. Dhara Jajal. We acknowledge the Dean, Physicians, Ophthalmologists and staff of the Pramukh Swami Medical College and Hospital, Karamsad, for the clinical diagnosis and help in the collection of blood samples. The authors thank all the participants for providing samples and co-operating during the entire study. We extend our gratitude to Dr. Harish Padh, Vice-Chancellor, Sardar Patel University, Vallabh Vidyanagar, for his valuable editorial help.

REFERENCES

1. Raman R, Rani PK, Rachepalle SR, Gnanamoorthy P, Uthra S, Kumaramanickavel G et al. Prevalence of diabetic retinopathy in India: sankara nethralaya diabetic retinopathy epidemiology and molecular genetics study report 2. Ophthalmology. 2009;116(2):311-318. DOI:10.1016/j.ophtha.2008.09.010
2. Patel S, Chen H, Tinkham NH, Zhang K. Genetic Susceptibility of Diabetic Retinopathy. Current Diabetes Reports. 2008; 8:257–262. DOI: 10.1007/s11892-008-0046-6
3. Tilton RG, Kawamura T, Chang KC, Ido Y, Bjercke RJ, Stephan CC et al. Vascular dysfunction induced by elevated glucose levels in rats is mediated by vascular endothelial growth factor. Journal of Clinical Investigation. 1997; 99:2192-202.
4. Qazi Y, Maddula S, Ambati BK. Mediators of ocular angiogenesis. Journal of Genetics. 2009; 88:495-515. DOI: 10.1007/s12041-009-0068-0
5. Clustering of long-term complications in families with diabetes in the diabetes control and complications trial. The Diabetes Control and Complications Trial Research Group. Diabetes. 1997; 46:1829–1839. DOI: 10.2337/diab.46.11.1829
6. Harris MI, Klein R, Cowie CC, Rowland M, Byrd-Holt DD. Is the risk of diabetic retinopathy greater in non-Hispanic blacks and Mexican Americans than in non-Hispanic whites with type 2 diabetes? A U.S. population study. Diabetes Care. 1998; 21:1230–1235. DOI: 10.2337/diacare.21.8.1230
7. Rema M, Saravanan G, Deepa R, Mohan V. Familial clustering of diabetic retinopathy in South Indian Type 2 diabetic patients. Diabetic Medicine. 2002; 19:910–916. DOI: 10.1046/j.1464-5491.2002.00820.x
8. Hallman DM, Huber JC, Gonzalez VH, Klein BE, Klein R, Hanis CL. Familial aggregation of severity of diabetic retinopathy in Mexican Americans from Starr County, Texas. Diabetes Care. 2005; 28:1163–1168. DOI: 10.2337/diacare.28.5.1163
9. Adamis AP, Miller JW, Bernal MT, Damico DJ, Folkman J, Yeo TK et al. Increased vascular endothelial growthfactor levels in the vitreous of eyes with proliferative diabeticretinopathy. American Journal of Ophthalmology. 1994; 118:445-50. DOI: 10.1016/S0002-9394(14)75794-0
10. Aiello LP, Avery RL, Arrigg PG, Keyt BA, Jampel HD, Shah ST et al. Vascular endothelial growth-factor in ocular fluid of patients with diabeticretinopathy and other retinal disorders. The New England Journal of Medicine. 1994; 331:1480-1487. DOI: 10.1056/NEJM199412013312203
11. Caldwell RB, Bartoli M, Behzadian MA, El-Remessy AEB, Al-Shabrawey M, Platt DH et al. Vascular endothelial growth factor and diabetic retinopathy: pathophysiological mechanisms and treatment perspectives. Diabetes/Metabolism Research and Reviews. 2003; 19:442–55. DOI: 10.1002/dmrr.415
12. Konopatskaya O, Churchill A, Harper S, Bates DO, Gardiner T. VEGF(165)b, an endogenous c-terminal splice variant of vegf, inhibits retinal neovascularization in mice. Molecular Vision. 2006; 12:626-32.
13. Simó R, Sundstrom JM, Antonetti DA. Ocular anti-VEGF therapy for diabetic retinopathy: the role of VEGF in the pathogenesis of diabetic retinopathy. Diabetes care. 2014; 37(4):893-899. DOI: 10.2337/dc13-2002
14. Ladomery MR, Harper SJ, Bates DO. Alternative splicing in angiogenesis: the vascular endothelial growth factor paradigm. Cancer Letters. 2007; 249:133–142. DOI:10.1016/j.canlet.2006.08.015
15. Abhary S, Burdon KP, Gupta A, Lake S, Selva D, Petrovsky N et al. Common sequence

variation in the VEGFA gene predicts risk of diabetic retinopathy. Investigative Ophthalmology and Visual Science. 2009; 50(12):5552-5558. DOI: 10.1167/iovs.09-3694

16. Al-Kateb H, Mirea L, Xie X, Sun L, Liu M, Chen H et al. Multiple variants in vascular endothelial growth factor (VEGFA) are risk factors for time to severe retinopathy in type 1 diabetes the DCCT/EDIC genetics study. Diabetes. 2007; 56(8):2161-2168. DOI: 10.2337/db07-0376
17. Churchill AJ, Carter JG, Ramsden, C, Turner SJ, Yeung A, Brenchley PE et al. VEGF polymorphisms are associated with severity of diabetic retinopathy. Investigative Ophthalmology and Visual Science. 2008; 49(8):3611-3616. DOI:10.1167/iovs.07-1383
18. Kangas-Kontio T, Vavuli S, Kakko S, Penna J, Savolainen ER, Savolainen M et al. Polymorphism of the manganese superoxide dismutase gene but not of vascular endothelial growth factor gene is a risk factor for diabetic retinopathy. British Journal of Ophthalmology. 2009; 93:1401–1406. DOI: 10.1136/bjo.2009.159012
19. Nakanishi K and Watanabe C. Single nucleotide polymorphisms of vascular endothelial growth factor gene intron 2 are markers for early progression of diabetic retinopathy in Japanese with type 1 diabetes. Clinica Chimica Acta, 2009; 402(1):171-175. DOI:10.1016/j.cca.2009.01.004
20. Yang X, Deng Y, Gu H, Lim A, Altankhuyag A, Jia W et al. Polymorphisms in the vascular endothelial growth factor gene and the risk of diabetic retinopathy in Chinese patients with type 2 diabetes. Molecular vision. 2011; 17:3088-3096.
21. Yang X, Deng Y, Gu H, Ren X, Li N, Lim A et al. Candidate gene association study for diabetic retinopathy in Chinese patients with type 2 diabetes. Molecular vision. 2014; 20:200-214.
22. Wilkinson CP, Ferris FL, Klein RE, Lee PP, Agardh CD, Davis M et al. Proposed international clinical diabetic retinopathy and diabetic macular edema disease severity scales. Ophthalmology. 2003;110(9):1677-1682. DOI:10.1016/S0161-6420(03)00475-5
23. Shi YY, He L. SHEsis, a powerful software platform for analyses of linkage disequilibrium, haplotype construction, and genetic association at polymorphism loci. Cell Res. 2005; 15(2):97-8.
24. Li Z, Zhang Z, He Z, Tang W, Li T, Zeng Z et al. A partition-ligation-combination-subdivision EM algorithm for haplotype inference with multiallelic markers: update of the SHEsis (http://analysis.bio-x.cn). Cell Res. 2009; 19(4):519-23.
25. Arjamaa O, Nikinmaa M. Oxygen-dependent diseases in the retina: role of hypoxiainducible factors. Exp Eye Res. 2006; 83:473–83. DOI: 10.1016/j.exer.2006.01.016
26. Renner W, Kotschan S, Hoffmann C, Obermayer-Pietsch B, Pilger E. A common 936 C/T mutation in the gene for vascular endothelial growth factor is associated with vascular endothelial growth factor plasma levels. Journal of Vascular Research. 2000; 37:443–448. DOI: 10.1159/000054076
27. Watson CJ, Webb NJ, Bottomley MJ, Brenchley PE. Identification of polymorphisms within the vascular endothelial growth factor (VEGF) gene: correlation with variation in VEGF protein production. Cytokine. 2000; 12:1232–1235. DOI:10.1006/cyto.2000.0692
28. Perrin RM, Konopatskaya O, Qiu Y, Harper S, Bates DO, Churchill AJ. Diabetic retinopathy is associated with a switch in splicing from anti- to pro-angiogenic isoforms of vascular endothelial growth factor. Diabetologia. 2005; 48:2422–2427. DOI: 10.1007/s00125-005-1951-8
29. Cartegni L, Wang JH, Zhu ZW, Zhang MQ, Krainer AR. ESEfinder: a web resource to identify exonic splicing enhancers. Nucleic Acids Research. 2003; 31:3568–3571. DOI: 10.1093/nar/gkg616
30. Smith PJ, Zhang CL, Wang JH, Chew SL, Zhang MQ, Krainer AR. An increased specificity score matrix for the prediction of SF2/ASFspecific exonic splicing enhancers. Human Molecular Genetics. 2006;15:2490–2508. DOI:10.1093/hmg/ddl171

Journal of
Oral Pathology & Medicine

J Oral Pathol Med (2017) 46: 106–111

wileyonlinelibrary.com/journal/jop

doi: 10.1111/jop.12468

Genetic profile of *PTEN* gene in Indian oral squamous cell carcinoma primary tumors

Sejal Shah[1], Dhara Jajal[1], Girish Mishra[2], Kiran Kalia[1,3]

[1]*Lab #103B, BRD School of Biosciences, Sardar Patel University, Gujarat, India;* [2]*Department of E N T, P. S. Medical College, Karamsad, Gujarat, India;* [3]*National Institute of Pharmaceuticals Education and Research (NIPER), Ahmedabad, Gujarat, India*

BACKGROUND: Phosphatase and tensin homolog (PTEN) is the tumor suppressor gene located on chromosome10q23.3. Genetic variations in the *PTEN* gene have been found in various sporadic tumors. However, petite is known about the genetic profile of the *PTEN* gene in oral squamous cell carcinoma (OSCC), which is the eighth most common neoplasm worldwide and leading cancer in India. The purpose of the present study was to determine the frequency of genetic variations in the tyrosine phosphatase domain of the *PTEN* gene in an Indian OSCC subset.

METHODS: We analyzed tyrosine phosphatase domain encoded by exon 5 of the *PTEN* gene in 59 OSCC primary tumors using PCR - direct genomic sequencing.

RESULT: We observed one somatic deletion mutation, IVS4-30delT in three OSCC patients; two of them were at an advanced stage of carcinoma. Moreover, we identified one SNP rs 35560700(C>T), in five OSCC patients with the late stage of oral carcinoma.

CONCLUSION: We identified 5% somatic mutational frequency in the intronic region of the tyrosine phosphatase domain of the *PTEN* gene; however, mutations were found absent in the coding region. Therefore, *PTEN* gene mutation is not a frequent event in the pathogenesis of OSCC in the targeted Indian cohort.

J Oral Pathol Med (2017) **46**: 106–111

Keywords: Indian population; mutation; oral squamous cell carcinoma; phosphatase and tensin homologue; single-Nucleotide Polymorphism

Introduction

The important footstep in most of the cancer development is the disarray of the protein function, which has a normal role in inhibition of tumor development, termed as tumor suppressors. PTEN is previously identified as tumor suppressor protein, phosphatase and tensin homologue deleted from chromosome 10/mutated in multiple advanced cancers (MMAC1/TEP1). *PTEN* gene encodes 403 amino acids and located on chromosome 10q23.3, which has a dual specificity phosphatase share the sequence homology with the protein tyrosine phosphatase (PTP) family and cytoskeletal proteins, tensin and auxilin. PTEN has been shown to be somatically deleted or mutated in a variety of cancers, such as breast cancers (4–6%), prostate cancers (35%), endometrial cancers (35–50%), glioblastomas (23–44%), and sporadic melanomas (43%), and suggested its function as a tumor suppressor gene (1). Mutations in the *PTEN* gene have been identified in several cancers, tumor lines, and inherited cancer syndromes such as, Cowden syndrome (CS) and Bannayan Ruvalcaba-Riley syndrome (BRR). The deactivating mutations in the coding region of the phosphatase domain abolished the phosphatase activity, suggesting that enzymatic activity is necessary for PTEN's ability to function as a tumor suppressor (2, 3).

Oral squamous cell carcinoma (OSCC) is the subset of head and neck squamous cell carcinomas (HNSCCs) ranked eighth most common human neoplasm worldwide and leading cause of mortality in the developing countries. It accounts for more than 260 000 cases and average 128 000 deaths every year globally and its overall incidence is 12.8/100 000 in men and 7.5/100 000 in women, in India (4). The concomitant use of tobacco in various forms is the prime risk factor, which has a great role in the development of OSCC. The statistical analysis of HNSCCs throughout the world has shown these cancers to be common in areas where tobacco and alcohol consumption is high (5). Betel quid chewing, the frequent and current form of tobacco chewing in the Asia-Pacific region is consisting areca nut, betel leaf, catechu, and slaked lime. About 10% of the world's population chew betel quid regularly (6). The Indian market is flooded with low-quality products with betel nut, tobacco, and combination of the variety of spices. All these goods are loaded with reactive oxygen species (ROS), catechu polyphenols, and slaked lime concentration. The oxidative stress and subsequent ROS generation leads to cell proliferation,

Correspondence: Kiran Kalia, Director, National Institute of Pharmaceuticals Education and Research (NIPER), C/o B.V.Patel PERD Centre, S.G.Highway, Thaltej, Ahmedabad - 380 054, Gujarat, India. Tel: +91 79 27439375, +91 9714618573, Fax: +91 79 27450449, E-mail: kirankalia@gmail.com
Accepted for publication May 23, 2016