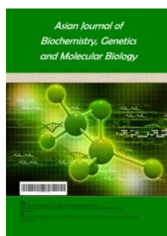

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Association of *LPAR1* Gene Variants with Type 2 Diabetes in Local Population

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Authors' contributions

This work was carried out in collaboration among all authors. Authors WR and BR designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors SZA, STQN and SAM managed the analyses of the study. All authors read and approved the final manuscript.

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ABSTRACT

Type 2 diabetes mellitus (T2DM) is the most prevailing worldwide health challenge of the 21st century and the 5th leading cause of death worldwide. About 90% of diabetic patients are diagnosed as having T2DM. *LPAR1* gene codes LPA protein that is involved in the regulation of many biological processes. In this study, we have investigated the association of single nucleotide polymorphism (SNP) of *LPAR1* gene variants rs494605 and rs558347 with T2DM in our local population. This association was analyzed by amplification of the target gene through Tetra ARMS PCR. The study involved 200 participants with equal ration of cases and controls. Both genetic variants of *LPAR1* rs494605 and rs558347 have allelic origin T/C. The allelic frequency of LPAR1 was calculated through the Hardy Weinberg Equilibrium. re found that in LPAR1 rs494605 mutant allele, C was 47% in cases compared to controls (39%) and in LPAR1 rs558347, heterozygosity allele (TC) was 46% compared to mutant allele C (13%), while wild T allele was 17% in cases. Many demographic and lifestyle risk factors were significantly associated with *LPAR1* gene variants. The heterogeneity of genetic variants with T2DM also showed a strong correlation with obesity, hormonal imbalance, and depression with *p*-value of 0.001, and 95% confidence interval. Smoking and alcohol consumption are major risk factors of T2DM. Variations in LPAR1 can be used as a biomarker and diagnostic tool for T2DM.

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Keywords: T2DM; LPAR1 gene; risk factors; genotyping.

1. INTRODUCTION

Type 2 Diabetes Mellitus (T2DM) is the metabolic illness of pancreatic islet cells that leads to dysregulation of insulin synthesis and pancreatic secretions [1]. Inappropriate insulin signalling disrupts its action and causes β-cells dysfunction and insulin sensitivity [2]. Insulin resistance indicates the reduced response of the liver, adipose tissues, and skeletal muscles to insulin receptors that stimulate hyperglycaemia by the failure of glucose uptake in peripheral tissues [3]. The number of patients has become doubled from the past two decades [4]. In Pakistan T2DM prevalence was 9.52% in 2004, the mean occurrence was 8.74% in 2007, and in 2009 the prevalence rate reached 19.21%. In 2010 only 10.85% of people were affected. About 10.95% of populations were affected by T2DM in 2013 [5]. The prevalence rate of T2DM in 2016 was 11.77% [6]. T2DM may result from genetic and environmental factors interact. Genetics, family history, race and ethnicity, age, gender are non-modifiable risk factors while environmental factors such as obesity, smoking, depression, alcohol consumption, physical inactivity, and dietary pattern are modifiable risk factors for pathogenesis [7]. Any change in insulin controlling genes affects metabolic pathways and stimulates T2DM [8].

In 2007, genome wide association studies (GWAS) has been recognized 38 vulnerable loci related to T2DM [9] that disrupt β-cell function, restrict insulin secretions and influence glucose homeostasis [10]. Gene translates Lysophosphatidic acid receptor 1 (LPAR1) that is involved in various biological and cytological processes [11] located at position 9q31.3 on chromosome 9 in humans and has 41kDa weight with 364 amino acids [12]. LPAR1 is expressed ubiquitously in cells throughout the body but its higher level is present in the placenta, brain, heart, small intestine, and colon [13]. But the *LPA* is a phospholipid that is an integral part of the plasma membrane and is involved in various cellular processes and signalling pathways. Free circulating LPA is produced by Autotaxin (ATX) enzyme [14].

But the *LPAR1* interact with G subunits (Gαq/11 and Gαi/o) and transduce signalling pathways through GTPases, Phospholipase C (PLC) and Phosphatidylinositol 3- kinase (PI3K) and subsequently activates cell proliferation, cell adhesion, migration, Ca^{2+} mobilization, myelination, cytoskeletal changes, and immune function [15]. The location of LPAR1gene is given in Fig. 1. In this study, our main objective is to find the association of SNPs of the LPAR1 gene (rs494605 and rs558347) with T2DM in our local population.

2. MATERIALS AND METHODS

2.1 Blood Sample Collection

This research is based on a case-control study. After getting approval from the ethical reviewer team of the Institutional Review Board (IRB) of the Institute of Molecular Biology and Biotechnology, BZU, Multan, a standard questionnaire was prepared. This form was filled and signed by patients who voluntarily participated in this investigation. Blood samples were collected from Nishtar Medical College and Hospital, Multan. This case-control study to verify T2DM susceptibility in the population of Southern Punjab, Pakistan, including two hundred subjects in which 100 were T2DM affected individuals as case-samples and 100 were non-affected healthy individuals as controls (Fig. 2). All the subjects willingly participated and were tested for the data collection on associated risk factors through the "standard interviewer-administered questionnaire".

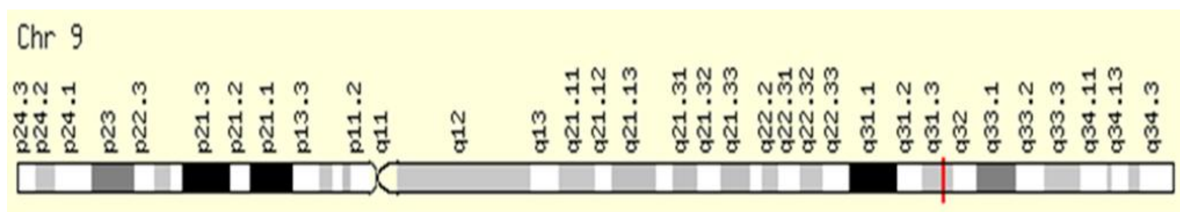

Fig. 1. Location of Lysophosphatidic acid receptor 1 (*LPAR1)* gene on chromosome 9 in *Homo sapiens* at position 9q31.3 (red line)

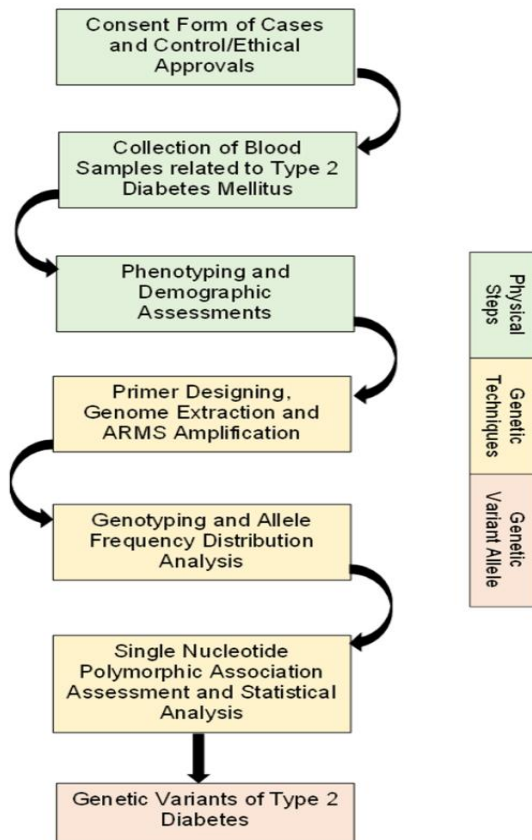

Fig. 2. Frame work of study to find genetic variants of type 2 diabetes mellitus

2.2 DNA Extraction, Quantification and Primer Designing

DNA extraction was done by in-organic protocol [16]. This procedure was followed by four steps as lysis and cell disruption, protein digestion, phase separation, and DNA precipitation. After extraction, DNA was quantified by using a UV-spectrophotometer (Perkin Elmer Lambda 25). Tetra ARMS primer software was used for primer designing. *LPAR1*gene variants primer sequence is given in Table 1.

2.3 Genotyping Analysis and Gel Electrophoresis

Tetra-primer ARMS PCR (tetra-primer amplification refractory mutation system polymerase chain reaction) was used for genotyping analysis. From Four primers, inner primers amplified the mutated allele. Recipe for 1x reaction mixture and thermos-profile for both variants are given in Tables 2 and 3. 2% Agarose gel was prepared to study bands and visualized under UV transilluminator. Two bands revealed a heterozygous genotype, while one band among the individuals under investigation displayed a homozygous genotype [17-20].

2.4 Statistical Analysis

Hardy Weinberg Equilibrium Analysis was done to find out the Genotypic Allelic frequency of LPAR1 variants. For Statistical analysis, SPSS v20 software was applied to identify the Chi-square (X2) and significance of demographic, genetic, and environmental factors with incidence of T2DM [21]. The Odds ratio (OD) and 95% confidence interval (CI) were measured by using the Online MedCalc calculator.

3. RESULTS

3.1 Single Nucleotide Polymorphism Analysis

The primary objective of this research design was to find the relationship of risk factors associated with T2DM and genetic polymorphism of *LPAR1* rs558347 and rs494605 by Tetra-primer ARMS PCR. For *LPAR1* rs558347, the product size is 196bp, 147bp, and 293bp (Fig. 3a). After PCR, got the outer band of 293bp, the inner band of 196bp showed homozygous ancestral allele T in the respective genotype. The inner band of 147bp represents homozygous mutant allele C. The observation of both 196bp and 147bp bands showed heterozygous allele TC. For *LPAR1* rs494605, the product size is 266bp, 202bp and 421bp (Fig. 3b). After amplification, it was observed a constant outer band of 421bp, the homozygous wild type T showed the band of 266bp, and homozygous mutant type C shows the 202bp band. The heterozygous TC shows both bands of 202bp and 266bp under UV transilluminator.

3.2 LPAR1 Genotypic Allelic Frequency

The Genotypic Allelic frequency of *LPAR1* rs494605 showed the ratio of mutant allele C in cases was 47% than 21% in control while ancestral T-allele ratio 66% was higher in control as compared to cases with 39%. Heterozygous TC genotype was greater in cases 14% as compared to controls 13%. SNP rs494605 showed a highly significant association in the sample population with a *p*-value of 0.002. In *LPAR1* rs558347, the ancestral genotypic ratio T was 41% in cases while in controls T was 49%. The rate of the mutant C allele was minutely

higher in the control population as in cases was 13% while in controls was 17%. The heterozygous TC genotype was 46% in cases and 34% in controls. The results showed a non-significant relationship of this variant with T2DM with a *p*-value of 0.206 in Table 4.

Table 1. Primer Sequence for *LPAR1* rs494605 and rs558347 variant

***LPAR1* Primers**	**Primer sequence**	**Designed T_M**
	rs494605	
Inner Forward (C-Allele)	378 TTTTTTAAATGAGTGCACAGAGAC 401	
Inner Reverse (T-Allele)	423 CATGTGCTTGCACTTTTATTCTA 401	57℃
Outer Reverse	578 GACCAAACATTAAAATCAAACATG 555	
Outer Forward	158 GAAATGTCATTAAATTACGGGTG 180	
Product size for C-Allele: 202bp, T Allele: 266bp, Outer Primers: 421bp		
	rs558347	
Inner Forward (T-Allele)	377TGAGCAATACCTTTTATCTGCCCAT 401	
Inner Reverse (C-Allele)	425GGAATTGGTTAAGAGCCTGTTGAAG 401	63℃
Outer Forward	279CATTGAAATGACTTGAATGCTAGTGC304	
Outer Reverse	571CAGTCATGTGTAGGGCATTATGGATA546	
Product size for C-Allele: 147bp, T-Allele: 196bp, Outer Primers: 293bp		

Table 2. 1x Reaction Mixture Recipe (7 µl) for SNP rs558347 & rs494605 of *LPAR1*

No. of Ingredients	**Reaction Mixtures Recipe (1x)**	**TOTAL (7 µl.)**
1	Template DNA	1µl
2	Outer Forward Primer	0.5 µl
3	Outer Reverse Primer	0.5 µl
4	Inner Forward Primer	0.5 µl
5	Inner Reverse Primer	0.5 µl
6	Master mix(2X M5 Hiper Plus Taq HiFi) PCR mix (with blue dye) MF 002-PLUS-01 (1ml)	2 µl
7	PCR Water	2 µl

Table 3. Tetra ARMS PCR Thermo Profile for *LPAR1* rs494605 and rs558347 variant

	Tetra ARMS PCR Thermo Profile	
PCR Steps	**Temperature (℃)**	**Time**
Initial Denaturation	95℃	5 minutes
35 Cycles		
Denaturation	94℃	30 seconds
Annealing	60℃ (rs558347) 56.5℃ (rs494605)	45 seconds
Extension	72℃	45 seconds
Final Extension	72℃	7minutes

Table 4. Genotypic Allelic frequency Analysis of *LPAR1*rs494605 and rs558347

			***LPAR1*rs494605**			
Genotypes	**TT**	**TC**	**CC**	**T frequency**	**C frequency**	**Significance**
Cases	39(0.39)	14(0.14)	47(0.47)	0.46	0.54	X^2 =16.92
Controls	66(0.66)	13(0.13)	21(0.21)	0.725	0.275	DF=2 **P=0.002**
			***LPAR1*rs558347**			
Genotypes	**TT**	**TC**	**CC**	**T frequency**	**C frequency**	**Significance**
Cases	41 (0.41)	46 (0.46)	13 (0.13)	0.64	0.36	X^2 =3.157
Controls	49 (0.49)	34 (0.34)	17 (0.17)	0.66	0.34	DF=2 **P= 0.206^{NS}**

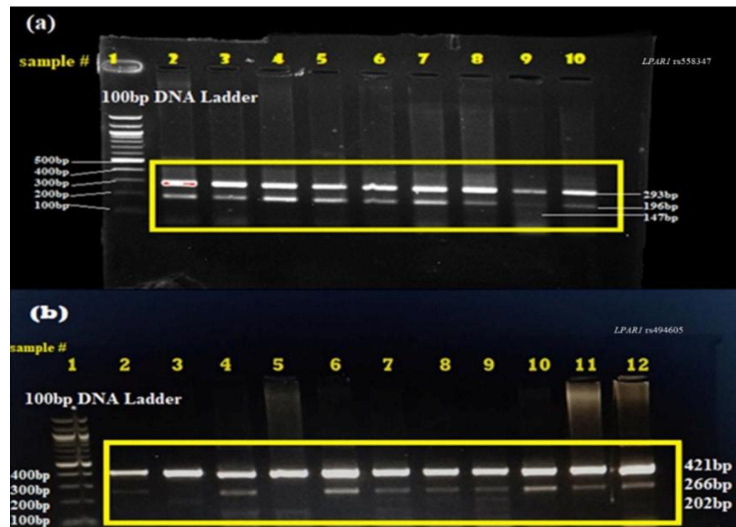

Fig. 3. (a) PCR product *LPAR1* rs558347, Lane1; 100bp DNA Ladder, Lane 2 to 10; Homozygous Wild TT allele 293bp & 196bp, Lane 9; Heterozygous TC allele 147bp, 196bp & 293bp (b) PCR Products for *LPAR1* rs494605, Lnae1: 100bp Ladder, Lane 7,8,9 Heterozygous TC allele 421bp, 266bp & 202bp, Lane 3 Homozygous mutant CC, 202bp& 421bp, Lane 2 and 1, 4,5,6,10,11,12; Homozygous wild TT 266bp & 421bp

3.3 Association of Demographic Factors with T2DM

In this study, it was analyzed the incidence of T2DM with different demographic, lifestyle, and behavioural risk factors. Study sample population contained 200 subjects as 100 cases and 100 controls. There were 75% male and 25% female in controls while 62% female and 38% male were in case population. Allelic frequency vs. gender distribution in cases and control samples was shown in Fig. 4. Statistical analysis of this factor showed a highly significant association (*p*-value 0.001). Diabetic history in family and inheritance of polymorphic loci make the generation more susceptible. In current study, result was found 53% of patients having a genetic history of T2DM while 47% of patients don't have any family history. This factor also represented a highly significant association (*p*-value 0.001). Age is an important risk factor as the T2DM exposure rate increases with age. In our sample population, the age group frequency was shown in Fig. 5. Result was found 63% of cases were between the ages of 31-50 years that showed a highly significant relationship of T2DM progression with this age group (*p*-value 0.001). Obesity also alters glucose index and disrupts insulin synthesis. It was calculated by measuring the Body Mass Index (BMI) of all subjects. Individuals with BMI ≥ 30 kg/m^2 were considered obese or overweight. We found 57% obese individuals in cases while in controls only 6% individuals were obese and 94% were non-obese that showed its highly significant association with risk of having T2DM (*p*-value 0.001). Cholesterol levels can also influence the metabolic pathway and increase the risk of T2DM. There were 9% controls and 22% cases found with an elevated level of cholesterol. While 91% controls and 78% cases were with normal cholesterol. It showed a significant association (*p*-value 0.011). Stress and depression also upturn the exposure to disease. In depressed people, blood sugar level increases due to cortisol hormone. In study population, result found 86% diabetic patients with depression. This was indicated a significant association (*p*-value 0.001). Smoking is a dynamic factor that decreases insulin functions and damages glucose homeostasis due to nicotine. In our case samples, it was found only 13% smokers, so this factor is non-significant (*p*-value 0.240). Hormonal imbalance is also an associated threat for T2DM. In study samples, 87% of diabetic patients had a hormonal imbalance that revealed the highly significant association of this factor (*p*-value 0.001). Alcohol and beverage consumption is also linked with the progression of T2DM in the western population. Alcoholic drinks are religiously restricted in our population and people don't confess about the use of drinks. It was found only one case with alcohol use. This represents the non-significant association of this factor in sample population (*p*-value 0.316) as shown in Table 5.

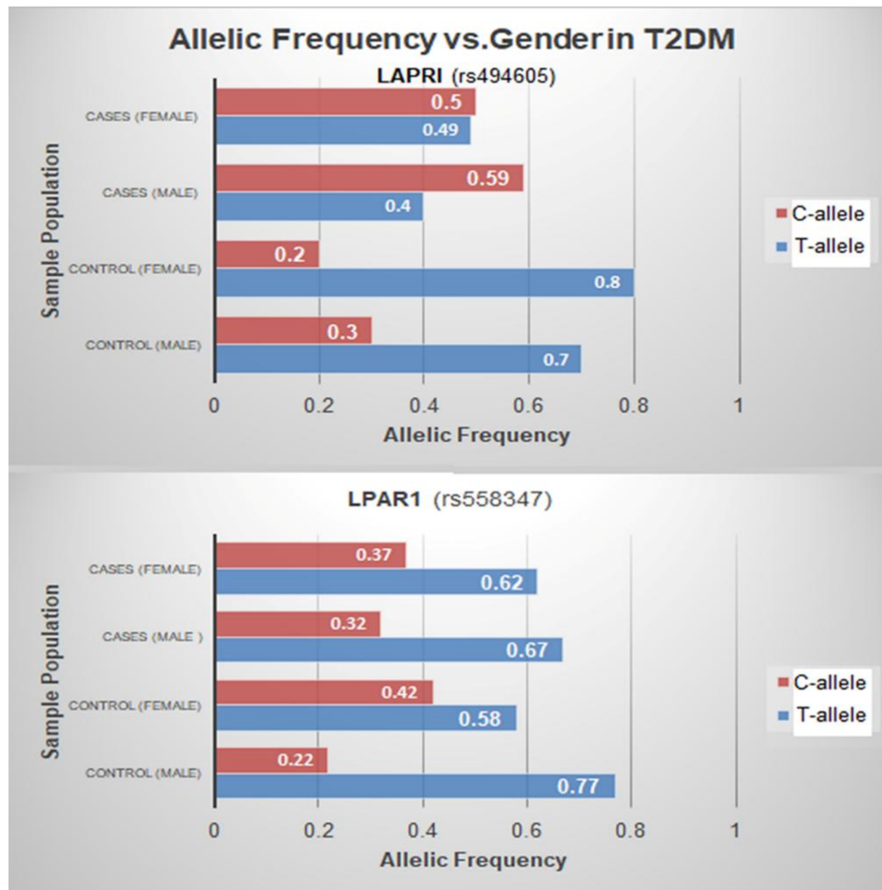

Fig. 4. Allelic frequencies vs. Gender distribution in sample population

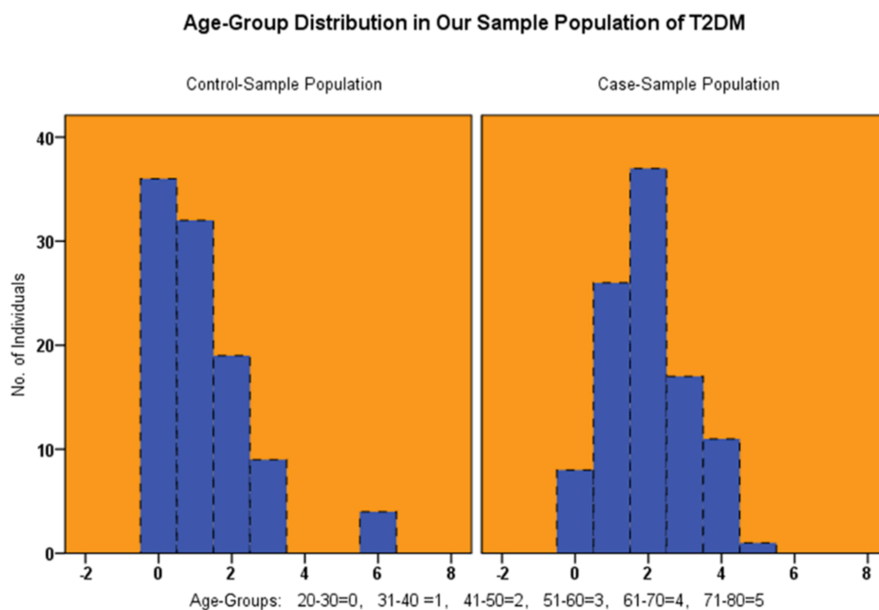

Fig. 5. Age group distributions in case and control population. Sample population was divided into age groups 20-30=0, 31-40=1, 41-50=2, 51-60=4, 71-80=5

Table 5. Demographic risk factors associated with T2DM

Factors	Category	Controls (N=100)	Cases (N=100)	Chi-sq	Df	P-value
Gender	Male	75 (75%)	38 (38%)	27.85	1	0.001**
	Female	25 (25%)	62 (62%)			
Family History	Yes	1 (1%)	53 (53%)	68.59	1	0.001**
	No	99 (99%)	47 (47%)			
Age	15-20	4 (4%)	0 (0%)	42.68	6	0.001**
	20-30	36 (36%)	8 (8%)			
	31-40	32 (32%)	26 (26%)			
	41-50	19 (19%)	37 (37%)			
	51-60	9 (9%)	17 (17%)			
	61-70	0 (0%)	11 (11%)			
	71-80	0 (0%)	1 (1%)			
Obesity	Yes	6 (6%)	57 (57%)	60.27	1	0.001**
	No	94 (94%)	43 (43%)			
Cholesterol	Yes	9 (9%)	22 (22%)	6.4516	1	0.011*
	No	91 (91%)	78 (78%)			
Depression	Yes	14 (14%)	86 (86%)	37.19	1	0.001**
	No	55 (55%)	45 (45%)			
Smoking	Yes	6 (6%)	13 (13%)	2.85	1	0.240^{NS}
	No	94 (94%)	87 (87%)			
Hormonal imbalance	Yes	14 (14%)	87 (87%)	106.59	1	0.001**
	No	86 (86%)	13 (13%)			
Alcohol user	Yes	0 (0%)	1(1%)	1.005	1	0.316^{NS}
	No	100 (100%)	99(99%)			

Not significant at p > 0.05=NS; Significant at p < 0.05=; Highly significant at p < 0.01=***

In this research, gene-environment interaction was studied by stratifying LPAR1 variants (rs494605, rs558347) with many reported risk factors for T2DM progression. The current study observed each risk factor vs. genotype to analyse the contingency significance in sample population. By this analysis, some variances in these results between the genotypic ratio of case and controls vs. associated lifestyle risk factor. The stratification between smoking vs. genotype of *LPAR1* rs494605 (*p*-value 0.308) and LPAR1rs558347 (*p*-value 0.461) showed a non-significant association with T2DM in population as showed in Table 6. When cholesterol was stratified with genotypes then elevated cholesterol was found non-significant in sample population as LPAR1 rs494605 (*p*-value 0.50) and LPAR1rs558347 (*p*-value 0.254) showed in Table 7. In sample, when individuals affected with hormonal imbalance were stratified with genotypes it showed no relation with the onset of T2DM as genotype LPAR1 rs494605 (*p*-value 0.67) and LPAR1 rs558347 (*p*-value 0.18) reported in Table 8. The differences started in research can be attributed to geographical, racial, and evolutionary variations in Southern Punjab population Pakistan.

4. DISCUSSION

Epigenetics, genetic predisposition, gene profiling, and genome-wide study helps to discover the novel SNP that involves in the pathogenesis of T2DM [22]. Any mutation in insulin regulatory genes impacts glucose metabolic pathways [8]. In this study, we have investigated the association of *LPAR1* gene variants (rs494605 and rs558347) with T2DM in local population by using Tetra ARMS PCR. *LPAR1* is located on the chromosome 9q31.3 in humans [12]. Polymorphic mutation existed in this susceptible loci disrupt glucose homeostasis [23]. The SNP present in intronic sequence characterizes as junk DNA and plays a functional role as a promoter. This enhancer control the expression of a gene and any mutation in this sequence causes the change in phenotype [24].

LPAR1 (rs494605 T/C) is an intronic variant in which the ancestral T allele is substituted with

Table 6. Polymorphisms of *LPAR1* gene variants: rs494605 & rs558347 associated with T2DM risk stratified by Smoking

			Smoking						
***LPAR1* rs494605**	**Smokers**				**Non smokers**				**Significance**
Genotypes	**Controls**	**Cases**	**X^2**	**P-value**	**Controls**	**Cases**	**X^2**	**P-value**	**Odd ratio (95% CI)**
TT	4	5	2.35	.308 NS	62	34	14.62	.001	
TC	1	1			12	13			0.4272/
CC	1	7			20	40			0.15- 1.17
***LPAR1* rs558347**									
TT	3	4	1.54	.461 NS	46	37	2.38	.306 NS	
TC	1	6			33	40			
CC	2	3			15	10			

Table 7. Polymorphisms of *LPAR1* gene variants: rs494605 & rs558347 associated with T2DM risk stratified by Cholesterol

			Cholesterol						
***LPAR1* rs494605**	**Elevated Level**				**Normal Level**				**Significance**
Genotypes	**Controls**	**Cases**	**X^2**	**P-value**	**Controls**	**Cases**	**X^2**	**P-value**	**Odd ratio (95% CI)**
TT	6	5	5.96	0.50 NS	60	34	9.56	0.008	
TC	1	2			12	12			0.3506/
CC	2	15			19	32			0.15- 0.80
***LPAR1* rs558347**									
TT	4	9	2.74	.254 NS	45	32	5.24	0.72 NS	
TC	4	10			30	36			
CC	1	3			16	10			

Table 8. Polymorphisms of *LPAR1* gene variants: rs494605 & rs558347 associated with T2DM risk stratified by Hormonal Imbalance

				Hormonal imbalance					
***LPAR1* rs494605**		**Affected**				**Non-Affected**			**Significance**
Genotypes	**Controls**	**Cases**	**X^2**	**P-value**	**Controls**	**Cases**	**X^2**	**P-value**	**Odd ratio (95% CI)**
TT	9	33	5.38	.067^{NS}	57	6	3.46	.176^{NS}	
TC	3	13			10	1			0.0243/
CC	2	41			19	6			0.01- 0.05
***LPAR1* rs558347**									
TT	1	37	7.93	.018 ^{NS}	48	4	4.90	0.85^{NS}	
TC	8	38			26	8			
CC	5	12			12	1			

mutant C allele by a transition (T > C) at 1250bp position (T1250C). The minor allelic frequency (MAF) is 0.44 and the highest population MAF is 0.55 taken from (ENSEMBL) [25]. In study population, allelic frequency distributional analysis had revealed that homozygous mutant C allele was higher (0.47%) comparatively wild T genotype (0.21%) in cases while in controls the ancestral allele T rate was high (0.66%) than mutant C (0.39%). This showed high disease risk development in our local population. *LPAR1* (rs558347 T/C) is present in the non-coding sequence of the genome. The wild T allele is altered with the C allele (T > C) at 319bp position (T319C) by the transition. The MAF for C is 0.29 and the highest population MAF is 0.47 from (ENSEMBL) [25]. This current study showed that the allelic frequency rate of the *LPAR1* (rs558347 T/C) mutant variant was high in controls (0.17%) as compared to cases (0.13%). This SNP of LPAR1 showed a non-significant association in population.

It has been studied that *LPAR1* comprises seven transmembrane domains that form three intracellular (ICL) and three extracellular loops (ECL) by modeling on the plasma membrane. The intracellular signal activates with the ICL2 region while the processing of receptor by cytoplasmic organelles is associated with ICL1 that allows the receptor to express on the surface of the cell [26]. ICL3 is the central region to attenuate signals. Generally, *LPAR1* receives extracellular ligands and activates downstream pathways to facilitate cellular response [27]. *LPAR1* interacts with G subunits and transduce signaling pathways that eventually activate cell proliferation, cell adhesion, migration, metastasis, necrosis, cytoskeletal changes, Ca^{2+} mobilization, and immune function [15].

Recent studies revealed that ATX- LPA levels correlate with regulatory effects on glucose homeostasis and insulin sensitivity [12]. LPA is a ubiquitous phospholipid that activates by binding G protein-coupled receptors and induces pathophysiological effects by multiple downstream signalling and overlapping expression pathways [28].

A literature study indicated that the LPA receptor plays a crucial role in hyperglycemia and diabetic nephropathy. Any mutation in the LPA receptor causes metabolic changes that increase ROS production and blood sugar level leads to vascular endothelial cell dysfunction. Subsequently, this effect activates several cellular signaling cascades including mitogen-activated protein kinases (MAPK), protein kinase C (PKC), transcription protein activator (JAK/STAT), transforming growth factor (TGF), and thereby inducing a cellular response through transcription factors (NF-κB). Many vasoactive factors enhance their fibrotic action in the progression of hyperglycemia and diabetic renal diseases [14].

The ATX- LPA signalling is also involved in several inflammatory and metabolic syndromes that include impaired glucose homeostasis, obesity, insulin resistance by promoting fibrosis [29]. Diseases that are linked with *LPAR1* are Spinal Stenosis and Pulmonary Fibrosis [30]. The elevated level of Serum LPA is also associated with essential hypertension. In the Chinese population, the *LPAR1* variant rs531003 is linked with hypertension [31]. *LPAR1* also acts as a biomarker for Prostate cancer [32,33]. In our study, *LPAR1* genetic variants showed a strong association with obesity or overweight, depression, cholesterol, and hormonal imbalance having (*p*-value 0.001). Smoking and alcohol drinking are major risk factors but in our study the association found is non-significant. *LPAR1* variants (rs494605 & rs558347) can be used as a therapeutic biomarker and diagnostic tool for T2DM.

5. CONCLUSIONS

From this study, results found that *LPAR1* showed a significant association with the commencement of T2DM in local population. Many demographic and lifestyle risk factors are significantly linked with *LPAR1* gene variants and T2DM progression. Advances in genome-wide profiling and epigenetic mechanisms lead to the discovery of more susceptible variants for T2DM.

ETHICAL APPROVAL

Ethical approval has been collected from the Institutional Review Board (IRB) of the Institute of Molecular Biology and Biotechnology, BZU, Multan.

CONSENT

All authors declare that written informed consent was obtained from the patient.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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