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Are we Predisposed to Type 2 Diabetes Risk: a Case-Control Study from Urban Population in Western India

Abstract

Background: Type 2 diabetes mellitus (T2DM) is driven by genetic and environmental factors. Sequence variants of candidate genes are known to predispose Indians to T2DM. 'Asian Indian Phenotype' is a cluster of abnormality described earlier; links possible genetic proneness of Indians. *PPARγ2* gene SNP rs1801282, a missense variant is associated with improved insulin sensitivity, reduced T2DM risk and adipogenesis. This polymorphism does not confer this benefit to Indian Asians as seen in Caucasians. *ADRβ3* gene SNP rs4994 produce variant receptor protein with altered affinity; is associated to development of dyslipidemia, obesity, insulin resistance (IR) and early T2DM onset. Combined influence of both these alleles on hemoglobin glycation, insulin resistance and dyslipidemia is not reported which is addressed in this case-control study from urban western Indian population.

Methods: T2DM subjects (n=506) and non-diabetic controls (n=544) largely from urban Hindu community were enrolled through consecutive sampling and studied for fasting plasma glucose (FPG), glycosylated hemoglobin (HbA1c), fasting insulin (FI), HOMA-IR, total cholesterol (TC), triglyceride (TG) and high-density lipoprotein cholesterol (HDL-C), Low-density lipoprotein cholesterol (LDL-C) and non-HDL-C through biochemical methods. SNPs were studied with restriction fragment length polymorphism (RFLP). Statistical analysis performed by Statav12 and IBM-SPSS included a variety of tests.

Results: T2DM subjects had higher FPG, HbA1c, HOMA-IR, TG and lower FI, HDL-C, LDL-C as compared to non-diabetic healthy individuals, while no differences in TC and non-HDL-C were seen in subgroups. The frequencies of reference and variant alleles of rs1801282 of *PPARγ2*and rs4994 of *ADRβ3* individually were similar in T2DM and controls.*PPARγ2* ref allele bearing T2DM subjects had a higher HbA1c and HOMA-IR in dyslipidemic as well as non-dyslipidemic than *PPARγ2*variant allele bearing counterpart. On allelic combination, presence of $PPAR\gamma2^{ref}$+$ADR\beta3^{va}$ was associated with high HbA1c and dyslipidemia than $PPAR\gamma2^{va}$+$ADR\beta3^{ref}$ allelic type. Taken together, reference allele of *PPARγ2*plus variant *ADRβ3*allelic type is likely to predispose to higher HbA1c, HOMA-IR and dyslipidemia.

Conclusion: Coexistence of reference allele of *PPARγ2* gene and variant allele of *ADRβ3* gene, dyslipidemia and obesity in an individual is likely to confer increased insulin resistance thereby increasing susceptibility to the development of T2DM.

Keywords: Type 2 diabetes mellitus; HbA1c; Dyslipidemia; *PPARγ2*; *ADRβ3*

Research Article

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Jayesh Sheth[1], Sunil Trivedi[1], Ankna Shah[1], Nutan Nabar[2], Navneet Shah[3], Premal Thakor[4], Rama Vaidya[2] and Frenny Sheth[1]

[1]*Department of Biochemistry and Molecular Genetics, FRIGE's Institute of Human Genetics, India*
[2]*Endocrine and Metabolic Disorders, Kasturba Health Society, Medical Research Centre, India*
[3]*Department of Diabetes and Endocrinology, Sterling Hospital, India*
[4]*Gujarat Diabetic Association, India*

***Corresponding author:** Jayesh J Sheth, Department of Biochemistry and Molecular Genetics, FRIGE's Institute of Human Genetics, FRIGE House, Jodhpur Gam Road, Satellite, Ahmedabad-380015, India, Tel: +91 79-26921414; 65128444; Fax: +91 79-26921415; Email: jshethad1@gmail.com

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Abbreviations: T2DM: Type 2 Diabetes Mellitus; *PPARγ2*: Peroxisome Proliferator-Activated Receptor γ2; *ADRβ3*: Beta-3-Adrenergic Receptor; IR: Insulin Resistance; FPG: Fasting Plasma Glucose; HbA1c: Glycosylated Hemoglobin; FI: Fasting Insulin; HOMA-IR: Homeostatic Model Assessment Insulin Resistance; TC: Total Cholesterol; TG: Triglyceride; HDL-C: High-Density Lipoprotein Cholesterol; LDL-C: Low-Density Lipoprotein Cholesterol; RFLP: Restriction Fragment Length Polymorphism; WHR: Waist to Hip Ratio; SNP: Single Nucleotide Polymorphism

Introduction

Type 2 diabetes mellitus (T2DM; OMIM 125853) is a complex metabolic disease triggered by the influence of numerous genetic and environmental factors [1,2]. Several studies have addressed the role of sequence variants of specific candidate genes [3,4]. Radha & Mohan [5,6]emphasized on a higher genetic predisposition of Indians to T2DM. Indians are reported to have a higher plasma insulin (compared to matched Europeans) [7], a higher WHR (waist to hip ratio) for any given BMI (compared to other ethnic groups) [8] and a greater insulin resistance (IR) [compared to other ethnic groups] [9-11]. Studies on migrant Indians have also shown a higher prevalence of T2DM (compared to indigenous population) [12-14]. Collectively, these studies point towards possibility of genetic proneness (genetic predisposition) of Indians to T2DM. In addition, fat tends to accumulate more in the abdominal region in Indians and therefore may be more prone to develop insulin resistance [10,15]. This cluster of metabolic

abnormality is collectively referred to as 'Asian Indian Phenotype' [16].

PPARγ2 , a candidate gene is predominantly expressed in adipose tissue and plays a vital role in insulin action and glucose homeostasis [17,19]. Moreover, it also act as a 'fatty acid sensor' with affinity towards free fatty acids [20,21]. SNP rs1801282 in the *PPARγ2* gene is a missense variant associated with improved insulin sensitivity, decreased risk of T2DM and adipogenesis; thus suggesting its role in glucose deregulation, dyslipidemia and obesity [17,18,20]. Furthermore, a recent meta-analysis of 41 studies showed that the presence of this variant allele was associated with T2DM (Meta-analysis; OR= 0.79, 95% CI= 0.66-0.95, I2= 69%) implicating its role in disease risk [22]. In addition, the Ala allele of this polymorphism was associated with a decreased risk of type 2 diabetes [23]. Moreover, Radha et al concluded that despite the frequency of the Ala allele at the PPARg-Pro12Ala locus was the same in individuals of South Asian descent, as in Caucasians, this polymorphism does not appear to improve insulin sensitivity or decrease risk for type 2 diabetes in South Asians (Asian Indians), as it does in Caucasians [5].

Likewise, *ADRβ3* is primarily expressed in adipose tissue and is implicated in lipolysis regulation [24]. SNP rs4994 of this gene is a missense variant that produces a protein (receptor) with altered affinity to norepinephrine.24Additionally, this variant has been shown to have an altered interaction with G-protein coupled receptors in adipocytes that may promote decrease in lipolysis resulting into development of dyslipidemia, obesity, insulin resistance (IR) and an earlier onset of T2D [24,25]. This may likely to be due to its influence on the glucose and lipid metabolism. However, inconsistent results on the association between the above variant alleles and T2DM risk may likely be due to the studies being carried out in individuals of different ethnicity [24,26]. The ADRB3 rs4994 polymorphism however, is reported to be associated with T2DM from Mexico city [27]. Moreover, Several past studies have connected this non-synonymous rs4994 polymorphism to T2DM, obesity, insulin resistance and hypertension [28-31].

Despite the availability of the association results with aforementioned variants for several ethnicity [18,19,25,26] no study has been reported the association between these variant alleles and T2DM in the western Indian population. Interestingly, interactions of alleles of *PPARγ2* (Pro12Ala) and *ADRβ3* (Trp64Arg) have been found to be related to obesity risk in Spanish children and adolescents as well as Mexican-American adults [32,33].

The current study takes into account the synergism of polymorphisms of (Pro12Ala in *PPARγ2* and Trp64Arg in *ADRβ3*) and aim to catch their influence on genetic proneness in a case-control study in largely Hindu urban Western Indian population by juxtaposing them to hemoglobin glycation, insulin resistance and dyslipidemia.

Materials and methods

Study population

A total of 1050 subjects were recruited for the study with 506 T2DM cases and 544 non-diabetic healthy controls from western India. They were enrolled after informed consent by consecutive sampling technique during April, 2012 to December, 2014 from the out-patient departments of several diabetes clinics and through weekly camps at two institutes (FRIGE, Ahmedabad and KHS's Medical Research Centre, Mumbai).The study were approved by the institutional Ethics committees of the institutions. The study participants were largely from Hindu community. The inclusion criteria for T2DM subjects were (a) age (≥25 years); (b) duration of diabetes (≥6 months from the date of diagnosis) and; (c) fasting plasma glucose levels (FPG ≥ 126.0mg/dl). Non-diabetic healthy controls were recruited with an inclusion criterion of (a) age (≥25 years); (b) HbA1c level ≤ 6.5% and; (c) fasting plasma glucose levels (FPG ≤ 110.0mg/dl). Subjects with Type 1 Diabetes, lactating and pregnant mothers, and those with concomitant chronic illness were excluded from the study.

Epidemiological questionnaire and sample collection

Each participant completed a standardized baseline questionnaire in person at each institute. The questionnaire included medical history, family history and demographic data. Blood samples were collected in fluoride, serum and EDTA vaccutainers in the morning (0800–1100 hrs) after 12 hours fasting for biochemical analysis [fasting plasma glucose (FPG), glycosylated hemoglobin (HbA1c), fasting insulin (FI), insulin resistance by HOMA-IR and lipid profile] and SNP genotyping. Serum samples were separated within 30-45 minutes of collection and stored at -20°C till further analysis.

Biochemical investigations

FPG and lipid profile including total cholesterol (TC), triglyceride (TG) and high-density lipoprotein cholesterol (HDL-C) were measured by colorimetric method with respective calibrator and biological standards by commercially available kits using an auto-analyzer BTS 330 (Biosystem, Spain)]. HbA1c was determined using affinity assay [NycoCard reader-II (Axis-Shield, Norway)] from whole blood. Serum FI levels were measured by Immuno Radiometric Assay [using commercial kit (Immunotech, France) with γ-counter system PC-RIA MAS (Stratec Biometrical System AG, Germany)]. Insulin resistance was calculated by the Homeostasis Model of Assessment-Insulin Resistance Index (HOMA-IR) [34]. Low-density lipoprotein cholesterol (LDL-C) and non-HDL-C were calculated by standard formula [LDL = TC - HDL-C - (TG/5) and non-HDL-C = TC - HDL-C]. The intra-assay coefficients of variation for lipid parameters were TC= 1.39%, TG= 0.50% and HDL-C= 1.67% whereas inter-assay coefficients of variations for aforementioned lipid parameters were 2.65%, 1.65% and 4.69% respectively.

SNP genotyping

Genomic DNA was extracted from whole blood by salting out method [35]. SNP rs1801282 and rs4994 were genotyped using restriction fragment length polymorphism (RFLP) PCR. The detailed PCR conditions are mentioned in supplementary Table 1. The amplified and digested PCR products were separated on 3% agarose gel and visualized under UV light based E-Gel imager (Life technologies, USA).

Supplementary Table 1

SNP ID	Primers (Forward & Reverse)	PCR Conditions			No. of PCR Cycles	Restriction Enzyme used for RFLP	Digested Product Allele	Product Size
			Temp. (°C)	Time (sec)				
						Method		
rs-1801282	5'-ACTCTGGGAGATTCTCCTATTGGC-3' 5'-CTGGAAGACAAACTACAAGAG-3'	Denaturation	94	20	28 Cycles	*HaeIII*	Wild Type (Pro12Pro)	132 + 23bp
		Annealing	56	20			Heterozygous Variant (Pro12Ala)	155 + 132+ 23bp
		Extension	72	20			Homozygous Variant (Ala12Ala)	155bp
rs-4994	5'-CGCCCAATACCGCCAACAC-3' 5'-CCACCAGGAGTCCCATCACC-3'	Denaturation	94	30	32 Cycles	*MspI*	Wild type (Trp64Trp)	152 + 58bp
		Annealing	69	30			Heterozygous Variant (Trp64Arg)	82 +70+ 58 +152bp
		Extension	72	30			Homozygous Variant (Arg64Arg)	82+70+ 58bp

Statistical analysis

The sample size (current study) yielded a margin error of 5% and confidence limit of 95% with an on-line calculator [36]. HbA1c values were mentioned as percentage of total hemoglobin and all other biochemical parameters were expressed as mg/dl. Student's t-test was conducted to compare the distribution of continuous variables between cases and controls. Correlation between the SNP, HbA1c and HOMA-IR was carried out using least squares linear regression. Hardy-Weinberg equilibrium (HWE) test for SNPs was performed in controls. Comparison of allele frequencies of SNPs between populations was carried out using Fisher's exact test. Binomial logistic regression was performed to yield odds ratios (ORs). ROC analysis to establish the cut-offs for HOMA-IR was performed in the study population. The significance threshold for two-sided type 1 error (P-value) in all tests was set at 0.05. False positive error/Type I error rate was 0.05. All statistical analysis was carried out in Stata v12 [for Macintosh OS (Stata Corp., College Station, USA)] and IBM-SPSS v19.0.

The cut-offs used for all the parameters for regression analysis were as follow: HbA1c >7% in T2DM subjects and ≤5.7% in non-diabetic control subjects [37]. HOMA-IR of ≤3.95 were considered as normal (as determined by ROC curve analysis). The cut-offs for lipids in accordance with NCEP guidelines [38] were: Cholesterol (220 mg/dl), triglyceride (150 mg/dl), HDL-C (40 mg/dl), LDL-C (130 mg/dl), non-HDL-C (160 mg/dl). One or more abnormal serum lipid biomarker concentration was regarded as dyslipidemia [38].

Results

Assessment of baseline anthropometric and biochemical characteristics

There were 50.95% males [N=535 (N=273 T2DM, N=262 non diabetic healthycontrols)] and 49.05% females [N=515 (N=233 T2DM, N=282 non diabetic healthy controls)] from amongst a total of 1050 study subjects. Base line anthropometric and biochemical characteristics are summarized in Table 1. The mean duration of diabetes amongst the T2DM subjects was 8.62 years (range: 6 months to 40 years) and had significantly higher FPG, HbA1c, HOMA-IR, TG and significantly lower FI, HDL-C, LDL-C as compared to controls (non-diabetic healthy individuals), while no differences were seen in TC and non-HDL-C amongst the two subgroups. T2DM subjects had also a higher BMI (Body Mass Index) and WHR (Waist to hip ratio) than controls.

Table 1: Anthropometric and biochemical characteristics of study subjects.

	T2DM (N=506)	Non-Diabetic Healthy Controls (N=544)
Anthropometric Data (Mean±SD)		
Age (Years)	56.88±10.44*	48.82±12.91
BMI (Kg/m^2)	27.28±5.14*	26.01±4.68
WHR	0.93±0.11*	0.91±0.07
Biochemical Parameters (Mean±SD)		
FPG (mg/dl)	139.62±48.37*	88.89±12.68
HbA1c (%)	8.14±1.77*	5.70±0.52
FI (µIU/ml)	10.77±6.52$	11.89±5.40
HOMA-IR	3.74±3.33*	2.67±1.54
TC (mg/dl)	176.92±51.42	180.98±46.49
TG (mg/dl)	141.28±73.55*	112.79±53.57
HDL-C (mg/dl)	53.86±16.66@	56.40±17.19
LDL-C (mg/dl)	94.32±49.28@	101.43±45.20
Non-HDL-C (mg/dl)	126.48±52.34	124.59±47.23

@ p <0.02; * p <0.001; $ p <0.002.

Allelic polymorphism rs1801282 of *PPARγ2* gene

The overall frequency of C allele (Pro, Reference allele) of rs1801282 of *PPARγ2*gene in the study cohort was 0.90 (81.41%, N=852) while frequency of G allele (Ala, Variant allele) was 0.10 (18.86%, N=198; Figure 1). There was a uniform distribution of both alleles in the study subjects by Hardy-Weinberg equilibrium (p=1.0, df=1). The Pro allele had a frequency of 0.91 (83.40%, N=422) in T2DM and 0.89 (79.04%, N=430) in controls while the Ala allele had a frequency of 0.09 (16.60%, N=84) in T2DM and 0.11 (20.96%, N=114) in controls respectively. Considering the lower frequency of Ala12Ala allele in T2DM (0.01%, N=5) and in control subjects (0.01%, N=7) respectively, subjects with Pro12Ala (heterozygous) and Ala12Ala (homozygous) were grouped together for further analysis. The frequencies of Pro (reference) and Ala (variant) allele were not different in the current study similar to Finnish, Spanish, North- and South- Indian subset populations; while in other populations, the frequencies were significantly different (Figure 1).

Allelic polymorphism rs4994 of *ADRβ3* gene

The overall frequency of A (Trp, Reference allele) allele rs4994 of *ADRβ3* gene in the study population was 0.91 (82.86%, N=870) while the frequency of G (Arg, Variant allele) allele was 0.09 (17.14%, N=180; Figure 2). Both alleles showed a uniform distribution in the study subjects by Hardy–Weinberg equilibrium (p=0.740, df=1). The Trp allele showed a frequency of 0.89 in T2DM (81.03%, N=410) and 0.92 in control subjects (84.56%, N=460) while Arg allele showed a frequency of 0.11 in T2DM (16.79%, N=85) and 0.08 in control subjects (14.89%, N=81). Considering the low frequency of Arg64Arg allele in T2DM (0.02%, N=11) and in control subjects (0.01%, N=3) respectively, those with Trp64Arg (heterozygous) and Arg 64 Arg (homozygous) were grouped together for further analysis. The reported frequencies of Trp (reference) and Arg (variant) allele were no different in the present study similar to Han Chinese (Beijing), Chinese in Denver (USA), Gujarati Indian (Houston), Japanese (Tokyo), Mexican (LA, USA),Masai in Kenya, Spanish and significantly different in other population subsets (Figure 1).

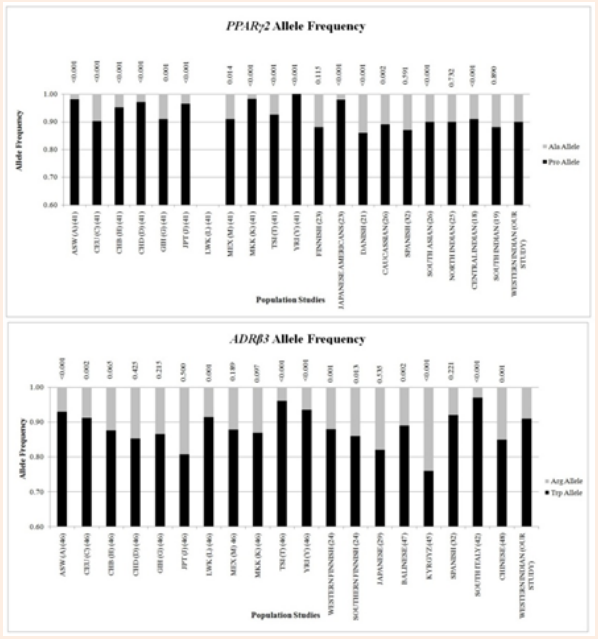


Figure 1: Comparison of allelic frequencies of rs1801282 of *PPARγ2*gene and rs4994 of *ADRβ3*gene.

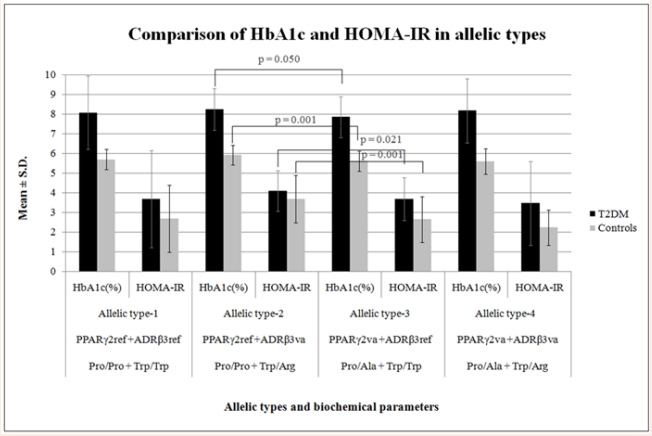


Figure 2: Comparison of allelic combinations of rs1801282 of *PPARγ2*gene and rs4994 of *ADRβ3* gene with HbA1c and HOMA-IR.

Association of allelic polymorphism of rs1801282 and rs4994 with T2DM risk

rs1801282and rs4994 were processed through logistic regression to identify their association with increased or decreased T2DM risk. However, we could not find any significant association of T2DM with variant allele of *PPARγ2* gene (OR= 0.85, 95% CI: 0.63-1.14, p=0.27), but the presence of the variant allele of *ADRβ3* gene was significantly associated with an increased risk of T2DM (OR= 1.48, 95% CI: 1.10-2.00, p=0.01).

Association of allelic polymorphism of rs1801282 and rs4994 to biochemical parameters

HbA1c and HOMA-IR were similar in diabetics bearing reference allele [(*PPARγ2*; Pro/Pro) or (*ADRβ3*; Trp/Trp)] or variant allele [(*PPARγ2* gene; Pro/Ala) or (*ADRβ3* gene; Trp/Arg)]. Control subjects with *PPARγ2* reference allele had significantly higher (p=0.04) HbA1c (though in the normal range) as compared to *PPARγ2* variant allele announcing a possibility of its diabetogenic role. In contrast, control subjects bearing *ADRβ3* reference allele had significantly lower HbA1c [though in the normal range (p=0.05)] than controls harboring variant *ADRβ3* allele suggesting a likelihood of its protective role (Table 2).

When presence or absence of dyslipidemia was added to both diabetics and non-diabetic controls, dyslipidemic diabetic individuals with reference *PPARγ2* allele had significantly higher HbA1c (p=0.048; Linear regression: r2=0.102, p<0.0010) and HOMA-IR (p=0.001) than variant bearing dyslipidemic diabetic individuals (Table 3). Amongst the non-dyslipidemic diabetics also, a similar observation was evident [higher HbA1c (p=0.001) and HOMA-IR (p=0.043) in *PPARγ2* reference allele bearing individuals than variant *PPARγ2* bearing individuals, (Table 3)]. Collectively these results point towards a diabetogenic role of *PPARγ2*reference allele than the variant allele amongst diabetic subjects. Control subjects on the other hand, did not show such difference in HbA1c or HOMA-IR.

Table 2: Influence of allelic polymorphism on HbA1c and Insulin resistance.

	PPARG2			
	T2DM		**Non-Diabetic Healthy Controls**	
	Reference Allele Pro/Pro (N=422)	Variant Allele Pro/Ala (N=84)	Reference Allele Pro/Pro (N=430)	Variant Allele Pro/Ala (N=114)
HbA1c (%)@	8.16±1.82	8.01±1.47	5.72±0.52*	5.62±0.06*
HOMA-IR@	3.76±2.45	3.66±2.67	2.68±1.63	2.62±1.13
	ADRB3			
	Reference Allele Trp/Trp (N=410)	Variant Allele Trp/Arg (N=96)	Reference Allele Trp/Trp (N=460)	Variant Allele Trp/Arg (N=84)
HbA1c (%)@	8.4±1.80	8.15±1.60	5.68±0.52**	5.80±0.52**
HOMA-IR@	3.68±2.53	4.00±2.64	2.68±1.60	2.63±1.16

@ Values expressed as Mean±SD

* p=0.04 ; ** p=0.05

Table 3: Influence of allelic polymorphism and lipid status on hemoglobin glycation and insulin resistance.

		T2DM		**Non-Diabetic Healthy Controls**	
		Dyslipidemia			
		PPARG2			
		Reference Allele Pro/Pro (N=225)	Variant Allele Pro/Ala (N=41)	Reference Allele Pro/Pro (N=205)	Variant Allele Pro/Ala (N=57)
HbA1c (%)	Mean ± SD	8.27±1.63*	7.74±1.24*	5.77±0.50	5.65±0.51
	R^2	0.102£	0.053	0.011	0.111
HOMA-IR	Mean ± SD	4.06±1.49**	3.24±1.32**	2.77±1.84	2.71±1.01
	R^2	0.004	0.006	0.072	0.172@
		ADRB3			

		Reference Allele Trp/Trp (N=209)	Variant Allele Trp/Arg (N=57)	Reference Allele Trp/Trp (N=220)	Variant Allele Trp/Arg (N=240)
HbA1c (%)	Mean ± SD	8.28±1.72	8.33±1.76	5.75±0.51	5.88±0.54
	R^2	0.098	0.019	0.087**	0.085
HOMA-IR	Mean ± SD	3.84±1.99$	4.61±1.02$	2.75±1.78	2.80±1.21
	R^2	0.057Ω	0.031	0.013	0.059
		No Dyslipidemia			
		PPARG2			
		Reference Allele Pro/Pro (N=197)	Variant Allele Pro/Ala (N=43)	Reference Allele Pro/Pro (N=225)	Variant allele Pro/Ala (N=57)
HbA1c (%)	Mean ± SD	8.17±1.68**	7.26±1.63**	5.68±0.53	5.58±0.54
	R^2	0.044	0.161	0.095£	0.024
HOMA-IR	Mean ± SD	3.64±1.74#	3.06±1.49#	2.61±1.41	2.53±1.24
	R^2	0.011	0.285Ω	0.012	0.113
		ADRB3			
		Reference Allele Trp/Trp N=201)	Variant Allele Trp/Arg (N=39)	Reference Allele Trp/Trp (N=240)	Variant Allele Trp/Arg (N=42)
HbA1c (%)	Mean ± SD	7.76±1.28	8.01±1.87	5.62±0.52	5.71±0.47
	R^2	0.018	0.392Δ	0.066∝	0.212
HOMA-IR	Mean ± SD	3.09±2.22	3.54±1.99	2.45±1.05	2.61±1.42
	R^2	0.072δ	0.232	0.026	0.054

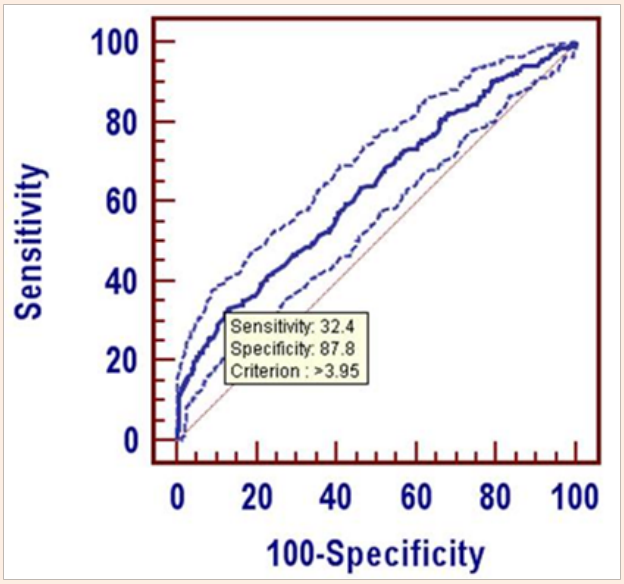
Linear Regression (LR) mentioned as R^2

P values: *0.048; ** 0.001; $0.005; # 0.043; £ 0.000; @ 0.040; Ω 0.019; Δ 0.002; ∝ 0.003; δ 0.012

ADRβ3 variant allele bearing dyslipidemic diabetic individuals exhibited a higher HOMA-IR (p=0.005; Linear regression: r2=0.057, p=0.019) than the reference *ADRβ3* allele bearing dyslipidemic diabetics suggesting a possible connection of variant allele to dyslipidemia and diabetogenicity; while such a difference was not seen in non-dyslipidemic diabetics. Control subjects did not show such difference (Table 3).

Assessment of rs1801282 and rs4994 allelic combinations with biochemical characteristics

rs1801282 of *PPARγ2* gene and rs4994 of *ADRβ3* gene allelic combinations yielded four allelic types [Allelic type-1: *PPARγ2*ref+*ADRβ3*ref (Pro/Pro+Trp/Trp); Allelic type-2: *PPARγ2*ref+*ADRβ3*va (Pro/Pro+Trp/Arg); Allelic type-3: *PPARγ2*va+*ADRβ3*ref (Pro/Ala+Trp/Trp); Allelic type-4: *PPARγ2*va+*ADRβ3*va (Pro/Ala+Trp/Arg)]. For assessment of interactions, we dichotomized biochemical parameters viz. HbA1c, HOMA-IR into normal/high and lipid status into dyslipidemia/non-dyslipidemia [HbA1c≥7.0% in T2DM and HbA1c≥5.7% in controls was regarded as high in accordance with IDF recommendations].22The cut-off of HOMA-IR (3.95) was determined on ROC analysis [specificity: 87.8% (95% CI: 84.7-90.4%); sensitivity: 32.45% (95% CI: 28.3-36.8); +LR: 2.66 (95% CI: 2.1-3.4); -LR: 0.77 (95% CI: 0.7-0.8); +PV: 77.7%; -PV 58.9%; Supplementary Figure 1].



Supplementary Figure 1

We compared the Mean±SD of HbA1c and HOMA-IR with

aforementioned four allelic combinations. A higher HbA1c and HOMA-IR was seen in allelic combination-2 as compared to combination-3 (HbA1c: T2DM p=0.050, Controls p=0.001) and (HOMA-IR: T2DM p=0.021, Controls p=0.001); Figure 2] suggesting a likelihood of association of *PPARγ2*ref and *ADRβ3*va alleles with poor glycation and insulin resistance in our study population.

When presence or absence of dyslipidemia was added to these allelic combinations, allelic type-2 bearing T2DM individuals but not control individuals had higher HbA1c (p=0.042) and HOMA-IR (p=0.002) than allelic type-3 (Figure 3A & B). Similarly, non-dyslipidemic individuals with allelic type-2 also demonstrated higher HbA1c (T2DM p=0.033; Controls p=0.001) and HOMA-IR (T2DM p=0.010; Controls p=0.001) than individuals (both diabetics and controls) with allelic type-3 again suggesting a likelihood of association of reference allele of *PPARγ2* gene and variant allele of *ADRβ3* gene to poor glycation and insulin resistance.

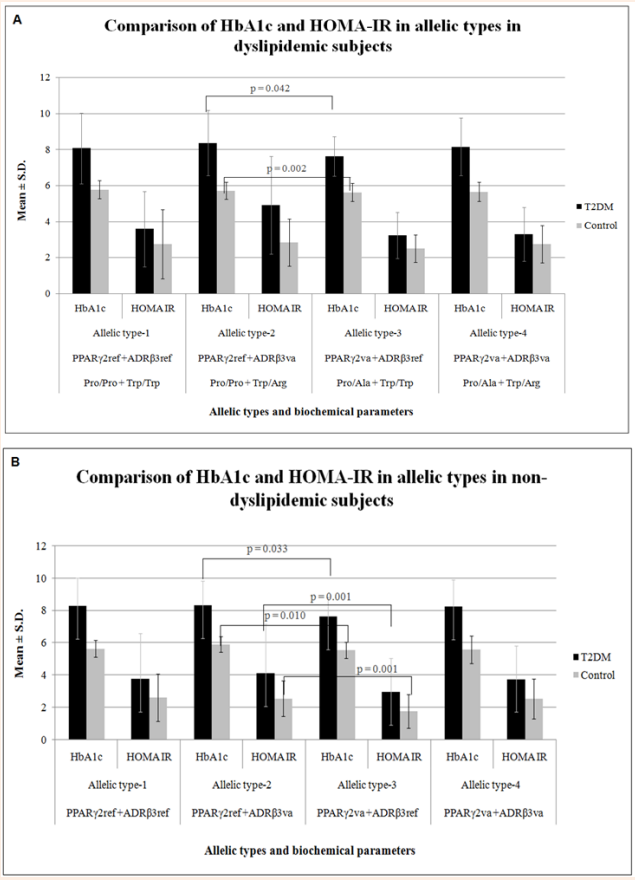


Figure 3: Comparison of allelic combinations of rs1801282 of *PPARγ2*gene and rs4994 of *ADRβ3*gene with HbA1c and HOMA-IR in dyslipidemic and non-dyslipidemic subjects.

We further elaborated our results with binary logistic regression model [where all these bifurcated (low/high; dyslipidemic/non-dyslipidemic) biochemical parameters were juxtaposed and compared to all six possible combinations of allelic types (Table 4)]. Again, presence of high HbA1c [p=0.034; LR (Likelihood Ratio): 4.559, p=0.033] and dyslipidemia [p=0.012; LR: 6.372, p=0.012] were significantly connected to allelic type-2. On the other hand, higher HOMA-IR was seen in all allelic combinations with type-4 [Higher Exp(B) between

i. allelic type-1 vs allelic type-4 (LR: 3.870, p=0.049),

ii. allelic type-2 vs allelic type-4 (LR: 3.675, p=0.055), and

iii. Allelic type-3 vs allelic tye-4 (LR: 5.808, p=0.016); Table 4] possibly suggesting a connection of *ADRβ3* variant allele to higher HOMA-IR.

Table 4: Interactions of allelic combinations with glycated hemoglobin, HOMA-IR and lipid status.

			Logistic Regression
			Exp(B)
Allelic type-1 vs Allelic type-2	HbA1c (%)	Normal	1.362
		High	1.296
	HOMA-IR	Normal	1.152
		High	0.977
	Lipid Status	No Dyslipidemia	1.145
		Dyslipidemia	1.379
Allelic type-1 vs Allelic type-3	HbA1c (%)	Normal	0.785
		High	0.909
	HOMA-IR	Normal	0.888
		High	0.736
	Lipid Status	No Dyslipidemia	0.897
		Dyslipidemia	0.772
Allelic type-1 vs Allelic type-4	HbA1c (%)	Normal	1.029
		High	1.17
	HOMA-IR	Normal	1.084
		High	857.944
	Lipid Status	No Dyslipidemia	1.168
		Dyslipidemia	1.111
Allelic type-2 vs Allelic type-3	HbA1c (%)	Normal	0.814
		High	0.452*
	HOMA-IR	Normal	0.685
		High	0.554
	Lipid Status	No Dyslipidemia	0.838
		Dyslipidemia	0.449**

Allelic type-2 vs Allelic type-4	HbA1c (%)	Normal	0.943
		High	1.255
	HOMA-IR	Normal	1.103
		High	25420.27
	Lipid Status	No Dyslipidemia	1.289
		Dyslipidemia	1.017
Allelic type-3 vs Allelic type-4	HbA1c (%)	Normal	1.967
		High	1.936
	HOMA-IR	Normal	1.614
		High	1.17E+09
	Lipid Status	No Dyslipidemia	1.983
		Dyslipidemia	2.688

* p=0.034; ** p=0.012;

Allelic type-1: PPARG2^{ref}+ADRB3^{ref} (Pro/Pro + Trp/Trp);

Allelic type-2: PPARG2^{ref}+ADRB3^{va} (Pro/Pro + Trp/Arg);

Allelic type-3: PPARG2^{va}+ADRB3^{ref} (Pro/Ala + Trp/Trp);

Allelic type-4: PPARG2^{va}+ADRB3^{va} (Pro/Ala + Trp/Arg)

Discussion

T2DM is the most common complex metabolic disorder influenced by genetic, environmental and lifestyle factors. Although the causes of T2DM cannot be pinpointed, many studies have shown a strong genetic link [20,39] to interactions of several candidate genes as an underlying etiology [25,26]. rs1801282 of nuclear receptor *PPARγ2* stimulates transcription of several genes implicated in adipogenesis, lipid metabolism and insulin signaling and might possibly affect insulin resistance. Alanine (G allele, variant allele) is shown to favor α-helix formation of *PPARγ2* gene product and activate amino terminal resulting into activation of ligand-independent transcription. Conversely, Proline (C allele, reference allele) modulates α-helix formation in a manner that predisposes to insulin resistance [20,21,23]. Thus, the variant allele is likely to have a protective role in insulin resistance, pathophysiology of T2DM and dyslipidemia. A study reported the relation of Ala allele of *PPARγ2* gene to lower the T2DM risk in Caucasians, and in over weight subjects it was found to be associated with lower insulin with increased insulin sensitivity [22]. Conversely, the Pro allele of *PPARγ2* gene is likely to increase insulin resistance and subsequent dyslipidemia in a meta-analysis [40]. The authors reported a frequency of Ala (variant allele) in the range of 8.0% to 51% in Caucasians (a subset of entire population). HAPMAP reported frequency of Ala (Variant) allele is in the range of 0 to 10%. We observed a frequency of Ala (variant) allele similar to GIH (Gujarati Indians in Houston, Texas, 9.09%) population of HAPMAP [41]. We observed that 79.80% (N=158/198) individuals bearing variant allele of *PPARγ2* had HOMA-IR below ROC cut-off supporting the previous reports of its likely association to insulin sensitivity. Conversely, high HOMA-IR (> ROC cut-off) was observed in 188/852 (22.06%) individuals bearing reference allele favoring its association to insulin resistance irrespective of the diabetic status. Two studies reported an association of Ala allele to reduced binding affinity to promoter elements and reduced ability to transactivate response elements of the responsive genes and improved insulin sensitivity [23] and reduced risk of insulin resistance with reduced TG levels in non-diabetics [21]. Though in the current study, we did not observe differences in incidence of dyslipidemia amongst diabetics and controls bearing Ala allele of *PPARγ2* gene. Mohan et al5 also concluded that despite the frequency of the Ala allele at the PPARg-Pro12Ala locus being the same in individuals of South Asian descent, as in Caucasians, this particular polymorphism does not appear to improve insulin sensitivity or decrease risk for type 2 diabetes in South Asians (Asian Indians), as it does in Caucasians.

Nonetheless, all dyslipidemic individuals of our study with Pro allele of *PPARγ2* gene had higher HbA1c and HOMA-IR than those with Ala allele (of *PPARγ2*gene) again favouring a likely association of this variant to predisposition for poor glycation and insulin resistance. A meta-analysis exhibited Ala allele of *PPARγ2* gene, to confer a risk reduction of T2DM by 21% [17]. Thus, there has been a consensus that *PPARγ2* variant allele confers 'protection' against T2DM and its likely manifestations in the form of reduced insulin resistance, reduced BMI and decreased lipid parameters. Conversely, presence of reference allele of *PPARγ2* gene has an opposite effect. Similarly, a study performed on South Asians (a subset of entire population) concluded that variant allele of *PPARγ2*gene does not offer any protective role [26].

ADRβ3 gene variant (rs4994) product, largely found in visceral adipocytes regulate lipolysis and mediate catecholamine induced cAMP activation by G-proteins [24]. The exact mechanism of *ADRβ3* gene in modulation of insulin secretion is not known, but glucose stimulated insulin secretion from pancreatic β cells is known to be potentiated by epinephrine. Lower levels of cAMP with variant allele of *ADRβ3* gene in pancreatic β cells may reduce insulin release [24]. Polymorphism of aforesaid gene (A allele, Trp64Arg) is shown to be associated to abdominal obesity and insulin-resistance [24,42,43]. Visceral obesity is also known to be associated with reduced FFA uptake and impaired insulin action that result into insulin resistance in skeletal muscle due to higher free fatty acids (FFA) or triglycerides [42,43]. Present study demonstrate significant association of variant allele rs4994 of *ADRβ3* gene with an increased risk of T2DM, most likely to be due to higher HOMA-IR in dyslipidemic T2DM patients. The variant allele of *ADRβ3* gene is also reported to be linked to insulin resistance in obese Italian males [44] and metabolic syndrome (obesity and low HDL-C) in Kyrgyz people [45]. HAPMAP reported frequencies of reference- (A allele) and variant (G allele) alleles of rs4994 of *ADRβ3* gene ranges from 80-96% and 3.9-19.1% respectively. We found a relatively higher frequency of variant allele (17.14%) similar to HAPMAP population of CHB (H) [Han Chinese in Beijing, China; 12.40%], CHD (D) [Chinese in Metropolitan Denver, Colorado; 14.67%], GIH (G) [Gujarati Indians in Houston, Texas; 13.36%], JPT (J) [Japanese in Tokyo, Japan; 19.19%], MEX (M) [Mexican ancestry in Los Angeles, California; 12.04%] and MKK (K) [Masai in Kinyawa, Kenya; 13.14%] [46-48]. This allele is

reported to be connected to insulin resistance in a meta-analysis of 31 studies [44].

While juxtaposing aforementioned alleles to link IR, T2DM and dyslipidemia, we observed a significantly higher HbA1c, increased IR and dyslipidemia in individuals with allelic type-2 compared to allelic type-3. This point towards a possibility that co-existence of reference allele of *PPARγ2* gene and variant allele of *ADRβ3*gene in an individual may predispose to develop biochemical deregulation connected to T2DM, poor hemoglobin glycation, dyslipidemia and subsequent metabolic syndrome. Likewise, co-existence of reference allele of *ADRβ3*gene and variant allele of *PPARγ2* gene is likely to be protective against T2DM. A similar finding has been reported by Moon et al. in Korean population [49].

The present study has also shown the likelihood of high HOMA-IR when allelic type-4 was present compared to type-1 to type 3 [as shown by high Exp(B) on logistic regression]. A similar report by Ochoa et al. also demonstrated higher BMI, serum leptin and insulin with co-existence of variants of *PPARγ2* gene and *ADRβ3*gene [32].

Coexistence of alleles in population is a natural phenomenon. Such allelic combinations along with life style predisposition may trigger epigenomic changes by increased DNA methylation of the genes and hold a clue to design tailored diagnostic, therapeutic and preventive efforts to control or subvert the disease. The functionality of the resultant proteins and their impact on metabolic pathways is greatly modified by epigenetic studies and protein-protein interaction. The allelic interactions reported here need further insight into epigenetics of these allelic combinations in pathophysiology of T2DM [32,33,50].

Conclusion

The present study demonstrate that coexistence of reference allele of *PPARγ2* gene and variant allele of *ADRβ3* gene together with dyslipidemia in subjects with T2DM is likely to confer poor hemoglobin glycation and increased insulin resistance; thus demonstrating a likelihood of T2DM predisposition in western Indian population.

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