Original Research Article

Association of non-synonymous +326 A>T and synonymous +258 G>A polymorphisms of omentin-1 gene (*ITLN-1*) among type 2 diabetes patients of Pakistan

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ABSTRACT

Omentin is an adipocytokine that has anti-diabetic property. Genetic variations in ITLN-1 may affect blood glucose level by inactivating Akt/PKB signaling pathway. The aim of this study was to evaluate the association between *ITLN-1* gene non-synonymous +326 A>T and synonymous +258 G>A polymorphisms with susceptibility to T2DM. Significant distribution observed in genotype frequency of +258G>A variants in T2DM patients and controls. Genetic models for co-dominant, dominant and recessive genotypes for +326 A>T and recessive genotype for +258 G>A were found capable of increasing the risk of T2DM. Allelic association analysis showed T-allele (+326 A>T) conferred higher risk of the diabetes. Results suggested that genetic variants of *ITLN-1* may act as possible molecular targets for early diagnosis of T2DM in future.

***Keywords*:** Omentin-1, synonymous +258 polymorphism, non-synonymous +326 polymorphism, allele specific-PCR, ARMS-PCR, Type 2 diabetes mellitus

INTRODUCTION

Diabetes Mellitus (DM) is a metabolic disorder that is caused by impaired glucose regulation. There are various types of DM but the most common one is Type 2 Diabetes Mellitus (T2DM). T2DM is an insulin resistance condition in which body cannot properly store and use energy, resulting in

hyperglycemia [1]. It had been estimated by International Diabetes Federation that 8.3 % of global population was affected with diabetes in 2019 which may rise up to 9.6 % by 2045 [2]. In 2019, more than 19.4 million cases of diabetes were reported in Pakistan with prevalence rate of 16.9 % [3]. Thus, management and cure of diabetes has become a global and local health concern. The progression of T2DM is chiefly caused by insulin resistance but there are some other factors such as environmental and genetic interactions, which can lead to diabetes development [1].

Omentin-1 (34kDa), also known as intelectin-1, is an adipokine, mainly expressed in visceral adipose tissue [4]. It has ability to prevent inflammation, atherogenesis, cardiovascular diseases and diabetes [5,6]. There are several functions of omentin-1 reported, such as it has the ability to enhance insulin sensitivity in human adipocytes to uptake glucose. Furthermore, presence and absence of insulin, does not affect omentin to activate protein kinase Akt/ protein kinase B pathways [7]. In the assessment of concentrations of omentin-1 in relation to T2DM, heterogeneity was significantly high, suggesting that certain undefined or unassessed variables may have been responsible [8]. Thus, for DM diagnosis, omentin-1 can be used as an efficient molecular biomarker.

The Omentin-1 is encoded by *ITLN-1* gene which is located on chromosome 1q23.3 [9]. Exon 4 of *ITLN-1* contains a nonsynonymous miss-sense genetic variation +326 A>T (rs2274907) and a synonymous genetic variation +258 G>A (rs2274908). The variation +326 A>T cause a change in nucleotide Adenine (A) to Thymine (T) at position 326 while +258 G>A shows variation from Guanine (G) to Adenine (A) at nucleotide position 258 [10]. Reduced level of omentin-1 serum has been reported in diabetic patients [11-14] but number of studies conducted to assess the effect of genetic variations in subjects with T2DM is very limited especially from Pakistani population.

The rationale of this study is to explore the current standpoint of single nucleotide polymorphisms +326 A>T and +258 G>A of *ITLN-1* gene with T2DM in Pakistan. Because of insufficient data, the management of DM appears as a great challenge to human health. This study will help to understand the role of *ITLN-1* as a possible marker in pathogenesis of disease.

MATERIALS AND METHODS

Study Subject and Ethical Approval

The study was permitted by ethical boards of The Karachi Institute of Biotechnology and Genetic Engineering (KIBGE), University of Karachi and Baqai Institute of Diabetology and Endocrinology (BIDE). An informed consent was taken from four hundred and forty cases with T2DM and equal number of controls.

Blood Sampling

After taking an informed written consent, five mL blood sample was collected in EDTA as an anticoagulant. For DNA extraction, standard phenol chloroform procedure was carried out [15]. The qualitative analysis of DNA was performed by 0.8 % Agarose gel electrophoresis and Nanodrop spectrophotometer was performed for DNA quantitative analysis.

Clinical Measurements

The clinical parameters of cases were recorded and Body Mass Index (BMI) of all participants was measured in kg/m^2. Following 12 h of overnight fasting, blood samples were obtained in EDTA tubes. Routine laboratory tests are analyzed in hospital laboratory, including HemoglobinA1c (HbA1c), serum Total Cholesterol (TC), Triglycerides (TG), High-Density Lipoprotein Cholesterol (HDL-C), Low-Density Lipoprotein Cholesterol (LDL-C), Random Blood Sugar (RBS), Fasting Blood Sugar (FBS), Systolic Blood Pressure (SBP) and Diastolic Blood Pressure (DBP).

Genotype Analysis

Genotype analysis of *ITLN-1* +326 A>T were carried out by tetra-amplification refractory mutation system polymerase chain reaction (T-ARMS-PCR) using thermal cycler (Biogener™, Hangzhou, China). The sense outer 5′-ACCCCTACCTTCCAGCCATCCC- 3′ and anti-sense outer 5′-CATGGGGCTGAAATGAACCCTCAGC - 3′ PCR primers were used to generate 403bp amplicon, sense outer and anti-sense inner 5′-TGCCGTCCCCCTCTGGGTAGT- 3′ primers generate a band size of 193bp for mutant T allele while for wild type A allele, sense inner 5′-GTCAGCAGGGCAGCAAAGCAGA- 3′ and anti-sense outer primers give 231bp amplicon. Genotype analysis of +258 G>A was carried out by Allele- Specific PCR (AS-PCR). The sense wild 5′-ACTTCCCACGCATGTCATTCTCG- 3′ and anti-sense common 5′-CTTTCTTGTCATGGGGCTGAAATGAA

C- 3′ primers were used to give 195bp amplicon for wild type G allele, while sense mutant 5′-ACTTCCCACGCATGTCATTCTCA- 3 and anti-sense common primers generate a band size of 195bp for mutant A- allele. The maximum volume 15µl PCR reaction mixture contains genomic DNA (50 ng), primers (20 pmol) and Dream-Taq Green master mix (2x) (Thermo Fisher Scientific, Waltham, MA, USA). PCR conditions for the amplification include initial melting of double stranded DNA at 95 °C for 4 min, followed by 39 cycles of denaturation at 94 °C for 60 sec, annealing at 58 °C for +326 A>T and 62 °C for +258 G>A for 40 seconds, and elongation at 72 °C for 60 sec with final elongation at same temperature for 5 min. The primers for both ARMS-PCR and AS-PCR were designed from Primer1 online software. Figure 1 depicted the amplified products for +326 A>T and +258 G>A that were observed on 2.5 % VisualaNA stained agarose gel under gel documentation system.

DNA Sequencing

For the validation of amino acid change in synonymous +326A>T polymorphism, AccuPrep® PCR Purifcation Kit (Bioneer, Deajeon, Korea) was used and PCR product purification protocol was performed as described by manufacturer. The purified products of PCR were then sequenced by MOLEQULEON, Auckland, New Zealand.

Statistical Analysis

For statistical analyses SPSS version 23, Chicago, IL, USA was used. Clinical parameters like age, BMI, TC, HDL-C, LDL-C, TG, RBS, FBS and HbA1C of cases and controls were compared and analyzed through Independent samples t-tests. Hardy-Weinberg Equilibrium (HWE) was calculated to check the +326 A>T and +258 G>A variations in *ITLN-1* is representative of our studied population by measuring difference between observed and predicted genotype frequencies in a population. Association analysis of genotypes with the disease was done through Pearson's chi square test and measured through odds ratio by MedCalc, an online tools for statistics [16]. A *p*-value of <0.05 was considered to be significant. Haplotype analysis and linkage disequilibrium plot was done by SHEsisPlus online software [17]. Molecular Evolutionary Genetic Analysis (MEGA) version 7.0 was used to analyze the obtained sequences [18].

RESULTS

This study involved 440 diagnosed T2DM patients (158 females and 282 males with means age 48.12±1.31 years) and 440 healthy

controls (160 females and 280 males with mean age 47.69±1.41years). The clinical characteristics of both cases and controls are shown in Table 1. The individuals with T2DM cases have significantly higher BMI and HDL *(p*<0.001), as well as total TC, TG, LDL, BPS and FBS level (*p*<0.0001) compared to controls. In contrast, T2DM patients have significantly lower values of DBP (*p*>0.05) when compared with control group.

Genotype distribution analysis of *ITLN-1* +326 A>T in T2DM patients and their corresponding controls are presented in Table 2. Results indicated a significant genotype distribution between +326 A>T variants of T2DM cases and their controls ($\chi 2$= 13.769, df= 2, *p*=0.001024). In addition, high percentage of heterozygosity i.e. AT genotype (61.8 %) was observed between T2DM patients when compared with control group (56.4 %). For genotype and allelic association analysis, data was analyzed assuming different genetic models and presented in Table 2. The results showed that codominant (O.R 2.9, CI= 1.5-5.8 *p*=0.0013), dominant (O.R 1.5, CI= 1.1-2., *p*=0.0032) and recessive (O.R=2.3, CI= 1.2-4.5, *p*=0.0080) genotypes of +326 A>T significantly increased the risk of T2DM. Additionally, multiplicative model for allele association analysis showed that T- allele of +326 A>T was significantly higher in T2DM patients compared with controls and it confers higher risk for the development of disease (O.R=1.3, CI= 1.1-1.6, *p*=0.0027).

Genotype distribution analysis of *ITLN-1* +258 G>A in T2DM patients and their corresponding controls are presented in Table 3. Results indicated a significant genotype distribution between *ITLN-1* +258 G>A variants of T2DM cases and their controls ($\chi 2$= 39.19, d*f*= 2, *p*<0.0001). Moreover, high percentage of homozygous wildtype GG (13.6%) and variant AA (13.2 %) was observed between T2DM patients when compared with control group (6 % and 4.5 % respectively). Genotype and allelic association analysis of +258 G>A variants showed that codominant (O.R 0.3, CI= 0.21-0.57 *p*<0.0001), dominant (O.R 0.39, CI= 0.24-0.64., *p*=0.0002) and over-dominant (O.R=0.31, CI= 0.21-0.46, *p*<0.0001) genotypes of +258 G>A significantly associated with protective role against T2DM. Additionally, multiplicative model for allele association analysis showed that T-allele of +258 G>A was not significantly associated with the risk of T2DM in our studied population. (O.R=1.01, CI= 0.84-1.22, *p*=0.8488).

To validate the amino acid change in +326 A>T variants protein, sequenced amplicon

were analyzed and compared with wild type sequences. Figure 2 confirms through the multiple sequence alignment that there was change from aspartate (D) to valine (V) at position 109 as a result of synonymous miss-sense polymorphism.

The multi-locus association test between +326 A>T (rs2247907) and +258 G>A (rs2247908) showing haplotype frequencies are presented in Table 4. The haplotype frequencies confirm the disease progression role of TG haplotype (O.R=2.66, CI= 1.67-4.235, *p*=0.000018) while protective role of AG haplotype (O.R=0.8, CI= 0.67-0.98, *p*=0.03) among T2DM patients. The multi SNPS analyses through linkage disequilibrium plot presented in Figure 3 suggested that targeted SNPs were in 70 % linkage disequilibrium with D'= 0.7 and R^2= 0.26.

Table 1. Demographic and clinical parameters of T2DM patients and control groups

Parameters	T2DM (n=440)	Controls (n=440)	*p*-value
Gender (Male/Female)	282 (64%)/ 158 (36%)	280 (63.6%)/ 160 (36.4%)	-
Age	48.0773 ± 0.473	47.69 ± 1.41	0.798
BMI	29.2503 ±0.2749	23.02 ± 0.349	<0.0001
TC	183.095 ±2.235	174.66 ± 2.182	0.007
TG	211.5227 ±7.3917	131.48 ± 4.092	<0.0001
HDL	38.7045 ±0.4319	42.38 ± 1.003	0.0008
LDL	102.077 ±1.581	126.030 ± 4.007	<0.0001
BPS	128.9±0.769	116.38±1.486	<0.0001
DBS	80.5±0.370	79.65±1.169	0.488
FBS	176.5±2.568	88.89 ± 1.344	<0.0001
HbA1c	9.2 ±0.09	-	-
RBS	235.8± 4.694	-	-

Table 2. Distribution and association analysis of *ITLN-1* +326 A>T

***ITLN-1* +326 A>T**					
Genotype		**T2DM (n=440)**	**Controls (n=440)**	**$\chi 2$ (d*f*=2)**	***p*-value**
AA		136 (31%)	178 (40.4%)	13.77	0.0010
AT		272 (61.8%)	248 (56.4%)		
TT		32(7.2%)	14 (3.2%)		
Genetic Models				**Odds Ratio (95% CI)**	
Co-Dominant	AA	136	178	1 (Ref.)	
	AT	272	248	1.43 (1.08-1.90)	0.0119
	AA	32	14	2.99 (1.53-5.82)	0.0013
Dominant	AA	136	178	1 (Ref.)	
	AT+TT	304	262	1.518 (1.15 to 2.00)	0.0032
Recessive	AA+AT	408	426	1 (Ref.)	
	TT	32	14	2.38 (1.25-4.54)	0.0080
Over-Dominant	AA+TT	168	192	1 (Ref.)	
	AT	272	248	1.25 (0.95-1.64)	0.1001
Multiplicative	A-allele	544	604	1 (Ref.)	
	T-allele	336	276	1.35 (1.11-1.64)	0.0027

Table 3. Haplotype analysis between *ITLN-1* +326 A>T and +326 A>T

Haplotype	**T2DM**	**Controls**		**OR (95% C.I)**	**p-value**
AA	168 (0.19)	184 (0.20)	0.909	0.89 (0.70-1.12)	0.34
AG	376 (0.42)	420 (0.47)	4.44	0.81 (0.67-0.98)	0.03
TA	270 (0.30)	250 (0.28)	1.09	1.11 (0.90-1.36)	0.29
TG	66 (0.07)	26 (0.03)	18.3	2.66 (1.67-4.23)	0.000018

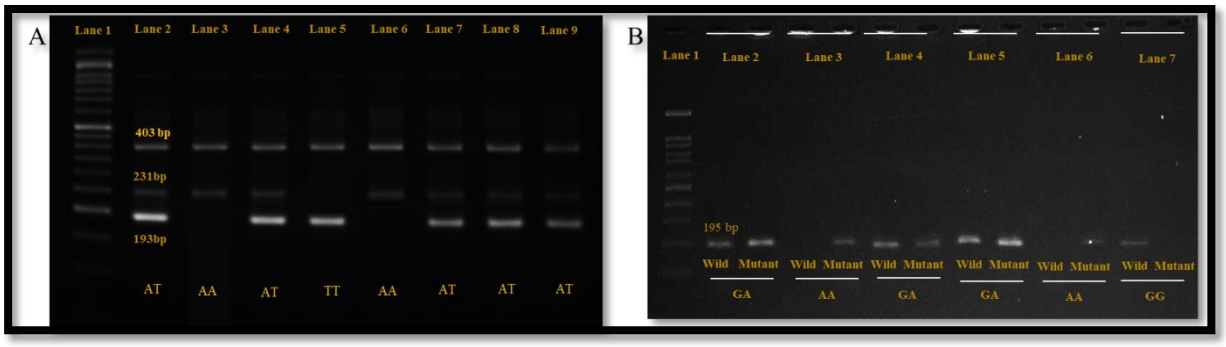

Figure 1. Agarose gel electrophoresis detection. (A) Represents *ITLN-1*+326A>T genotypes by ARMS-PCR analysis. (B) Represents *ITLN-1* +258 G>A genotypes by AS-PCR analysis.

Figure 2. Multiple sequence alignment change in amino acid aspartate (D) to valine (V).

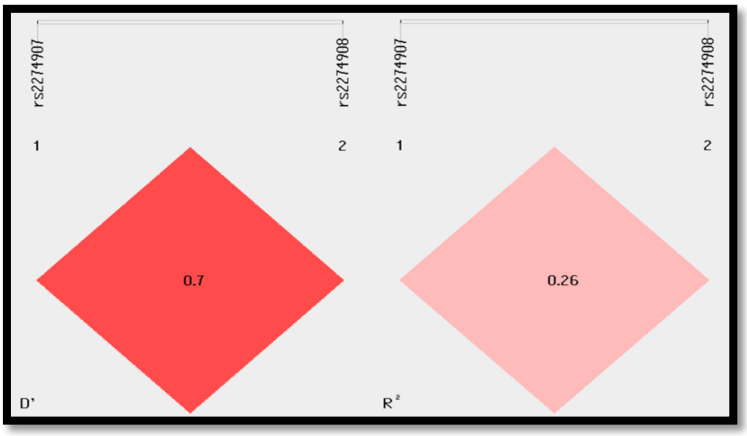

Figure 3. Linkage disequilibrium (LD) block *ITLN-1* +326 A>T (rs2274907) and +258G>A (rs2274908).

Table 4. Distribution and association analysis of *ITLN-1*+258 G>A genotypes

***ITLN -1* +258 G>A**					
Genotype		**T2DM (n=440)**	**Controls (n=440)**	**χ2 (d*f*=2)**	***p*-value**
GG		60 (13.6%)	26 (6%)	39.19	< 0.00001
GA		322 (73.2%)	394 (89.5%)		
AA		58 (13.2%)	20 (4.5%)		
Genetic Models				**Odds Ratio (95% CI)**	
Co-Dominant	GG	60	26	1 (Ref.)	
	GA	322	394	0.35 (0.21-0.57)	<0.0001
	AA	58	20	1.25 (0.63-2.49)	0.5137
Dominant	GG	60	26	1 (Ref.)	
	GA+AA	380	414	0.39 (0.24-0.64)	0.0002
Recessive	GA+GG	382	420	1 (Ref.)	
	AA	58	20	3.18 (1.88-5.39)	0.0001
Over-Dominant	GG+AA	118	46	1 (Ref.)	
	GA	322	394	0.318 (0.21-0.46)	0.0001
Multiplicative	G-allele	442	446	1 (Ref.)	
	A-allele	438	434	1.018 (0.84-1.22)	0.8488

DISCUSSION

Genetic variation in *ITLN-1* linked to insulin resistance as it may affect the glucose uptake by dysfunction of insulin receptor substrate, deactivation of Protein kinase B and AMP protein kinase [19]. Other risk factors such as increased BMI, lipid accumulation or altered plasma omentin-1 level in association of diabetes mellitus were widely studied but the outcomes of these studies are uncertain [20]. Therefore the rationale of current study is to investigate genetic variations associated with *ITLN-1* gene among Type 2 diabetes mellitus patients of studied Pakistani population.

In this study, +326 A>T of *ITLN-1 gene* was investigated in patients with T2DM and control groups. It was found that T-allele of +326 A>T is associated with high risk of T2DM. Different genotype models suggest that dominant (AA vs AT+TT), co-dominant (AA vs AT) and over- dominant (AA+TT vs AT) genotypes confers higher risk for the pathogenesis of T2DM, as the odds ratios are higher than reference value i.e. 1. Similar to current findings, It has been reported in Iranian population that variants of +326 A>T were linked with the greater risk of T2DM [21]. Comparably, a previous case-control study conducted in Germany did not determine a significant allelic distribution of +326 A>T SNP in T2D patients relative to healthy controls. [10]. Another study conducted in Poland also did not show any significant relationship between *ITLN-1* polymorphism +326 A>T and T2DM with other metabolic syndrome risk [22, 23].More recently, Rathwa et al., failed to find any association of +326 A>T variants with T2DM [13]. The studied single nucleotide polymorphism (SNP) +326 A>T A/T is present on exon 4 of *ITLN -1*. In this miss-sense SNP, the codon GAC which codes for aspartic acid is replaced by codon GTC which codes for amino acid valine. As aspartic acid is negatively charged amino acid and valine is hydrophobic amino acid, therefore it might be possible that this substitution can inactivate or alter omentin-1 protein function. However a study suggested that this amino acid change has nonthreatening effect on omentin-1 protein [13]. Thus future functional studies are

required for omentin protein for advance investigation.

The significant distribution of *ITLN-1* +258 G>A genotype between T2DM cases and controls did not show significant allele association in progression or control of diabetes. Moreover, co- dominant (GG vs AA), dominant (GG vs GA+AA) and over-dominant models (GG+AA vs GA) showed significant negative association of +258 G>A with T2DM, while recessive model (GA+GG vs AA) represented significant positive association with the advancement of disease. This demonstrates that A allele will only increase the risk of T2DM when it will be in homozygous form. Interestingly, there is no study present on +258 G>A that claims any association with diabetes. In this synonymous SNP (sSNP), change in codon from CGT to CAT at position 86 does not replace amino acid histidine which is the consequence of genetic code degeneracy. Such variations can disrupt the gene expression at post-transcriptional and translational levels by modifying micro-RNA binding site, transfer-RNA binding, and pre-mRNA splicing. It also affects secondary structure and stability of mRNA, co-translational folding and thus the structure of the protein. Hence these factors might trigger functionally significant changes [24].

Many researchers investigated the association of +326 A>T polymorphism in the *ITLN-1* gene and/or Omentin-1 levels with various diseases, rheumatoid arthritis [25], psoriasis [26], osteoporosis[27], coronary artery disease (CAD) [28,29 & 30], breast cancer [31] and systemic lupus erythematosus [33]while there is only single study found that investigated association of +258 G>A with CAD [32].

Haplotype analysis between +326 A>T and +258 G>A display a significant interaction of TG haplotype which suggested that presence of T allele and absence of A allele can serve as marker for diabetes susceptibility. In this study linkage disequilibrium plot suggested that the two studied SNPs of *ITLN-1* were in high association (D'= 0.7) and can be inherited as haplotype.

The association analyses between *ITLN-1* polymorphisms +326 A>T and +258 G>A with T2DM improves our insight into the effect of gene variants on clinical phenotype. Such studies may be useful in predicting T2D and associated morbidity in normal individuals with family history of diabetes. However, these observation needs validation by large sample size studies for Pakistan as well as other populations.

CONCLUSION

This study is possibly the preliminary study conducted in Pakistan that has recognized *ITLN-1* +326 A>T and +258 G>A genetic variations in T2DM. Therefore, examining this polymorphic domain can be helpful in anticipating T2D and the associated complications in healthy subjects. Consequently, conducting genome-wide association research on more gene polymorphisms linked to insulin resistance is an effective strategy for diagnosis of patients with diabetes and preventing diabetes. Therefore, this study will help to understand the role of *ITLN-1* as a possible marker in pathogenesis of these diseases.

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