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Research Article

Association of the Gene *FTO* Single Nucleotide Polymorphism, rs9939609 with Type 2 Diabetes Mellitus in Pakistani Cohort

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Abstract: To date, inconclusive data is available about the insight of the *FTO* gene variant with type 2 diabetes mellitus. T2DM is a chronic disease and a rising problem worldwide. Its complications lead to an increase in the burden of mortality specifically in lower and medium-income countries. Genome-wide association studies have spotted many genetic loci that are related to T2DM and validate the complicated polygenic traits. Many variants of different genes including *FTO* are associated with T2DM hence, this study was designed to inspect and unfold obscure data in South Asians. The main objective of present study is to identify the relation of *FTO* intronic variant rs9939609 with T2DM in Karachi-based Sindhi population of Pakistan. Total recruited individuals were grouped as diabetic cases and controls. Out of the total recruited subjects, genotyping was done on 152 samples using T-ARMS PCR however, demographic and clinical data were recorded of all individuals. The results showed that the frequency of variant genotypes in the diabetic case group was 11 % for AA, 45 % for AT and 44 % for TT though, the frequency of the lethal allele (T) was 34 %. These outcomes concluded, rare T allele frequency is higher among diabetic cases as compared to controls and provides the contribution from the Pakistani population to support the previous controversial findings. This study concluded *FTO* gene-single nucleotide polymorphism, rs9939609 is associated with T2DM but still, it is a growing need to do further studies on T2DM susceptible genes with different polymorphisms to recognize targets in the field of pharmacogenomics for clinical implementation.

Keywords: Genome-Wide Association Studies, Type 2 Diabetes Mellitus, Single Nucleotide Polymorphism, Fat mass and obesity-associated gene

1. INTRODUCTION

Type 2 diabetes mellitus (T2DM) is escalating enormously and is indicated by beta cell dysfunction and insulin resistance. Its complications proceed to enhance the burden of mortality globally according to World Health Organization [1]. A decade ago, it was estimated 3.96 million deaths in adults due to diabetes mellitus however, the number raised to million deaths later in five years which is almost equal to a single death in every second. In adults till 2030, a 20 percent rise in diabetes mellitus will occur in developed countries but an alarming 69 percent upsurge of diabetes mellitus is predicted in developing countries [2]. In accord with International Diabetes Federation (IDF) Diabetes Atlas, Asia is one of the major and highly prevalent area of T2DM epidemic and is propagating rapidly in low- as well as in middle-income countries [3]. After India and China, USA was listed as the third highest country of patients with T2DM thereby, 25 cents of health expenditure are utilized in its treatment. This global estimation focuses on the severity of the T2DM pandemic [4]. Moreover, T2DM also increases with the rise in obesity specifically in childhood however, complications initiate in adulthood [5].

The gene, Fat mass and obesity associated (*FTO*) is most commonly coupled alongside obesity, an excessive fat mass suffice to elevate the possibilities as well as hazards of mortality and morbidity [6]. It is recognized as the key vulnerable factor for various non-communicable illnesses including coronary

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heart disease, cardiovascular disease, hypertension and the T2DM. In addition, it predisposes to varied mental complications or physical infirmities. Forty-four percent of diabetic cases are accountable for the condition of overweight and obesity [7]. Among other non-communicable ailments, T2DM is most convincingly related to obesity. The occurrence of obesity-associated diabetes is likely to increase to three hundred million by the year 2025 [8]. Body mass index is a measurement for obesity and defines the anthropometric features of height and weight, though, not a sensitive metric to identify early fat deposits in adults and childhood [9].

Genome-wide association studies (GWAS) reported several genetic loci linked along obesity and T2DM that validate the complicated polygenic traits. Many variants of various genes are associated with T2DM and obesity. The gene *FTO* has been studied in numerous European and South Asian populations however, the results of studies are still unclear [10].

However, one recent study has been conducted among the people of Bangladesh and showed a positive association of *FTO* with T2DM [11]. On the other hand, another recent study on the Indian population showed no direct association of *FTO* with T2DM [12]. Moreover, no similar recent study has been done in Pakistan and to date, no previous data is available on Pakistani Sindhi origins of people. Hence, the present study was intended to further inspect and unfold obscure data of *FTO* in our targeted population which has not been studied before. The aim and objective of this analysis were to identify the involvement of *FTO* intronic variant rs9939609 with T2DM in the Karachi-based Sindhi population of Pakistan.

2. MATERIALS AND METHODS

2.1 Subject Recruitment

A total of 1666 samples representing adults from the largest metropolitan city, Karachi of Pakistan were analyzed. All recruited subjects belong to the Sindhi cohort of the Pakistani population, sum of 1504 people were enlisted in this study after gaining individual consent and ethical clearance from GC University, Pakistan and the remaining 162 samples were taken from a repository of the Department of Biological and Biomedical Sciences (DBBS), Aga Khan University. All the subjects were grouped into type 2 diabetic cases and diabetic controls. 152 samples from the recruited individuals were selected for variant genetic analysis.

2.2 Sample Collection

Blood sampling was done from the antecubital vein as per standard protocol. Participants' recruiting proforma was filled as per inclusion and exclusion criteria [13].

2.3 Physical and Biochemical Measurements

The quantitative anthropometric non-invasive measurements for the BMI of each recruited member were taken. The waist circumference (WC) was measured as per standard protocol with the tape. The tape was placed halfway in between the last bottom rib and the upper top of the hipbone. Later, the hip circumference (HC) was also measured with a measuring band positioned at the broadest area of the buttocks. In WC and HC measurements, the tape snugged around and was not constricting as per WHO steps. Another parameter, the waist-to-hip ratio (WHR) was computed by the formula as; WHR is waist circumference/hip circumference in centimeters [14, 15]. Systolic and diastolic blood pressure measurements were done by using a mercury sphygmomanometer and a stethoscope [16]. Biochemical profiling including fasting plasma sugar (FPS), total cholesterol (TC), as well as low/high density lipoprotein levels (HDL-C/ LDL-C), was done through commercial kits, Abcam USA [13].

2.4 DNA Extraction and Genotyping

Genomic DNA extraction of each sample was done as per the Promega kit protocol. A DNA purity check of each sample was done through Nanodrop-ND1000 by a company Thermo Fisher Scientific. All participants were genotyped for the *FTO* variant, rs9939609 by an economical inhouse polymerase chain reaction (PCR) technique, the tetraprimer - amplification refractory mutation system, T-ARMS PCR. The allele-specific fragments were generated by using specific primers as shown in Table 1. The primers were designed through the biocomputational tool, Primer3Plus.

This tool is freely available on the internet and can search the weblink by an online search engine.

PCR was performed in an absolute volume of 10 ul. The one reaction mixture contained 150 ng genomic DNA. Moreover, 1XPCR buffer, the 3.0 mM $MgCl_2$ besides, 1.0 mM dNTPs were added. In addition, outer forward or reverse (OF / OR) and inner forward or reverse allele-specific primers (IF / IR) were added with the concentration of 10 picomoles each. The enzyme, Taq polymerase was added with total units of 1.5 in each reaction (Promega, USA).

PCR was carried out by thermal cycler, Mastercycler Eppendorf program as per optimized conditions. Table 2 shows the allele (T/A) specific products generated by PCR reaction. Formerly, these products or amplicons were processed on one percent horizontal agarose gel electrophoresis and finally, spotted on the gel documentation system (Bio-Rad) under the ultraviolet light.

2.5 Statistical Analysis

All data was statistically investigated by using Statistical Package for Social Sciences, SPSS version IBM 20. Hardy Weinberg equilibrium (HWE) test was used to attain the deviation of population. Basic demographic and biochemical characteristics data were analyzed by mean ± standard deviation. In addition, association of SNPs with T2DM and obesity was statistically analyzed via logistic regression analysis. The association was also calculated after adjustment of BMI and obesity related trait, waist circumference. Linear regression was also used to test the factors effect size.

3. RESULTS AND DISCUSSION

3.1 Demographic and Biochemical Variables

Characteristics of study population, anthropometric and biochemical data were recorded and are showing in the table 3. All the controls were matched to T2DM cases on the basis of age and gender.

3.2 HWE, Genotype and Allele Frequencies of *FTO* Variant rs9939609

The genetic analyses were carried out by taking frequencies of all *FTO* variant genotypes and alleles in control subjects as well as in T2DM cases. All the individuals were tested and the data is provided in Table 4.

The genotype frequencies in control category individuals were 9 % for AA, 42 % for AT and 50 % for TT however, the frequency of lethal allele (T) in control category individuals was 29 %. HWE test was done only in controls to confirm the homogenous standardized population. Results indicate observed and expected value (χ^2 = 0.79; p-value = 0.07) of controls which is non-significant, specified control subjects are descending in consonance with HWE.

The occurrence of genotypes in the diabetic case

Table 1. Specific primer sequences for *FTO* variant, rs9939609

Primers abbreviations	Sequences 5'-3'	Len (bp)
OF	CAGTTCCAGTCATTTTTGACAGC	23
OR	TGTTCAAGTCACACTCAGCCTCT	23
IF	TCCTTGCGACTGCTGTGAATATA	23
IR	ACAGAGACTATCCAAGTGCATCTCA	25

Len (bp) = Length of sequence in base pairs outer forward or reverse (OF / OR) and inner forward or reverse allele-specific primers (IF / IR)

Table 2. Primer specific PCR products

Primers	Amplicon type	Amplicon size (bp)	Genotypes
OF and OR	non-specific	446bp	TA
OF and IR	for allele A	212bp	AA
IF and OR	for allele T	148bp	TT

Table 3. Demographic and biochemical characteristics of study participants

Variables	Diabetic Control subjects mean (SD, if specified)	T2DM case subjects mean (SD, if specified)
Total count	1281	385
Age in years	51.1 (10.7)	53.5 (10.7)
Percent male	46.3	40.0
Systolic blood pressure (mmHg)	135.6 (22.8)	143.4 (24.2)
Diastolic blood pressure (mmHg)	85.9 (12.8)	88.3 (12.6)
FPS (mmol/l)	5.3 (0.6)	10.6 (4.0)
Weight in Kgs	63.4 (13.8)	66.4 (15.0)
Height in cm	1.6 (0.1)	1.6 (0.1)
BMI Kg/m2	25.2 (5.2)	26.7 (5.6)
Waist-circumference in cm	88.1 (12.0)	93.2 (11.7)
T.C in mmol/L	4.8 (1.0)	5.0 (1.2)
T.G in mmol/L	3.0 (0.80)	3.1 (0.9)
LDL-C in mmol/L	3.0 (0.80)	3.1 (0.9)
HDL cholesterol in mmol/L	1.1 (0.3)	1.0 (0.3)

Values are presented as means (SD), where specified Total Cholesterol, T.C; Fasting plasma sugar, FPS; Triglycerides, T.G

Table 4. *FTO* SNP rs9939609 genotype and allele frequencies

Genotypes	Controls	Cases
AA	109(0.09)	43(0.11)
AT	536(0.42)	173(0.45)
TT	636(0.50)	169(0.44)
Total	1281	385
Minor allele frequency (A)	0.29	0.34
HWE (Chi-square, $\chi 2$)	0.07(degree of freedom df=1)	-
p-value	0.79	-

set individuals was 11 % for AA, 45 % for AT and 44 % for TT nevertheless, the frequency of lethal allele was 34 %.

Type 2 diabetes susceptible gene, *FTO* SNP rs9939609 genotyping of recruited subjects was done by T-ARMS PCR. Figure 1 is an electrophoretogram showing lanes from 1 to 12. The gel was prepared with agarose with a percentage of one. Subsequently, the prepared gel was assembled on gel electrophoresis apparatus (Sigma). The comb used in assembling the gel comprised of 12 wells. Fragments sizes are displayed on the right of Figure 1. The non-specific DNA fragment is of 446 base pairs, the wild type A-allele specific fragment is of 212 base pairs and the rare T-allele fragment is of 148 base pairs. All were visualized under the ultraviolet light through the Bio-rad gel documentation system.

3.3 Association of *FTO* rs9939609 with T2DM in Total Study Subjects

The results were evaluated by logistic regression analysis and showed the relationship of *FTO* genotype with T2DM, each copy of the A-allele expands the hazard of diabetes trait with an odds ratio, 1.21 with limit of 95 % confidence interval. These associations remained very similar although insignificant p-value once adjusting models for BMI (odds ratios of 1.17 with limit of 95 % confidence interval) or waist-circumference (odds ratios

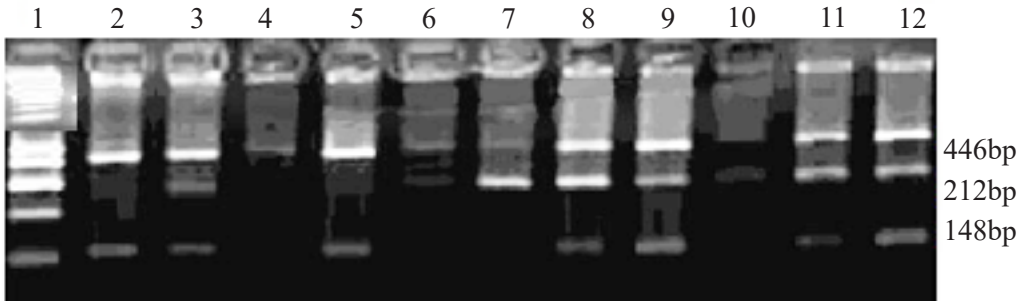

Fig.1. PCR results of *FTO* variant rs9939609 on type 2 diabetic cases in Pakistani population. The figure is an electrophoretogram showing lanes from 1 to 12. The gel was prepared by agarose with the percentage of one. Subsequently, the prepared gel was assembled on gel electrophoresis apparatus (Sigma). The comb used in assembling the gel comprised of 12 wells. Fragments sizes are displaying on the right of figure which was compared with the ladder fragments in lane 1. The non-specific DNA fragment is of 446 base pairs, the wild type A-allele specific fragment is of 212 base pairs and rare T-allele fragment is of 148 base pairs. All were visualized under the ultraviolet light through Bio-rad gel documentation system

of 1.15 with limit of 95 % confidence interval). In the study, results also indicated a connection between *FTO* genotype and FPS within individuals in the cohort. This also remained alike, even next adjustments for BMI or WC, though statistically insignificant (Table 5).

3.4 Association of *FTO* rs9939609 with BMI and Obesity in Total Study Subjects

In this study, a significant increase in BMI was observed with growing numbers of risk-allele in the study, 0.52 kg/m^2 (95 % CI; p value, 0.006). In addition, a similar outline was detected for waist-circumference, with a minor single allele effect of 1.20 cm (95 % CI; p value, 0.007). Moreover, when BMI was divided into two contrasting traits of abnormal weight (overweight or obesity) and normal weight as per WHO Asian criteria, the data indicated insignificant results in effect of normal BMI and with another measure of obesity, waist circumference (WC) however, abnormal BMI in the study showed a per allele effect with an odds ratio of 1.21 (95 % CI; p value, 0.02) as shown in Table 6.

The previous studies analyzed the involvement of rs9939609 variant with the disease T2DM in

Table 5. Association of *FTO* variant rs9939609 in accumulation with factors (age, gender, BMI, WC) with T2DM in total study subjects by linear regression analysis

Phenotypic variables	Models	beta Co-efficient	P-value
Diabetes* (odds ratio)	I (Age, gender)	1.21 (1.02–1.45)	0.03
	II (Age, gender, BMI)	1.17 (0.97–1.41)	0.09
	III (Age, gender, WC)	1.15 (0.94–1.41)	0.17
Fasting glucose (mmol/l) (β-coefficient)	I(Age and gender)	0.24 (0.02–0.46)	0.03
	II (Age, gender, BMI)	0.21 (−0.01 to 0.43)	0.06
	III (Age, gender, WC)	0.19 (−0.03 to 0.41)	0.09

Table 6. Odds ratio of *FTO* variant rs9939609 with adiposity in study subject by logistic regression analysis

Phenotypic Variables	Risk allele effect size 95%CI	P value
Normal BMI (kg/m2)	0.52 (0.15–0.89)	0.006
Waist circumference (cm)	1.20 (0.33–2.07)	0.007
Overweight/obesity	1.21 (1.03–1.41)	0.02

Asian populations. These studies have revealed results that are not consistent and still debatable [17, 18]. This study report is the contribution from Pakistan to explore the association of the *FTO* common variant with T2DM. The results of this study generate a main validation of *FTO* variant rs9939609, located on the chromosome 16. The wet-lab genetic analysis of *FTO* was done and the data showed promising results that *FTO* gene variant is strongly connected with type- 2 diabetes in our selected Sindhi cohort. Through statistical analysis, it was inferred that *FTO* variant in selected cohort from Karachi population is strongly associated with common disease T2DM. The results of this study are in parallel and similar to few other Asian and European studies [18-21].

T2DM is a chronic metabolic syndrome that has an impact on millions of individuals worldwide and is considered as a major public health concern in our society. There are several risk factors including the modifiable and non-modifiable, yet both are associated with diabetes. All the genetic factors that account for T2DM are non-modifiable however positive lifestyle incorporation such as exercise and controlled healthy diet can lead a way to either prevent or delay T2DM [22].

Another study showed this *FTO* rs9939609 risk allele is less recurring in East Asian individuals, 12.6 % [23] in comparison to the European individuals and West African individuals risk allele frequency, 45 % and 52 % respectively [24, 25]. Moreover, few studies showed significant association between *FTO* rs9939609 variant and T2DM [26] in contrary, other studies did not find any relationship between these two [27].

Another study has been conducted in North Indians to find out the common *FTO* variants association with T2DM but showed inconsistent results. Hence, *FTO* rs9939609 variant out of eight variants showed no association with T2DM [28]. The recent similar study was conducted among the Bangladesh people in the year 2023, their results revealed significant association with T2DM [11].

Another study in Vietnamese population was done in the year 2022 and reported that *FTO* variant is a predictor for future T2DM [29]. The present study is coinciding with these previous findings and could be used as a basis for future association research involving larger populations. In the present study, *FTO* polymorphism showed higher minor allele frequency in cases than controls. Moreover, the major power and strengths of this *FTO* polymorphism study is, the selected cohort was founded on similar population deprived of any genetic admixture. In addition, the diabetic cases were identified created on fasting glucose test. The *FTO* gene is popular for obesity and performs a polygenic effect as reported by genome wide association studies (GWAS). The selected gene variant has shown significant association of *FTO* risk-allele with BMI, a risk marker for obesity [30].

The limitations of the study are additional statistical tests have to be done by adjustments and making various models to evaluate the potential confounding variables including obesity-related traits as well as clinical patterns. Besides, more supportive studies in the Pakistani population are required with an increased sample size.

4. CONCLUSION

The current study uncovered significant associations of *FTO* gene SNP, rs9939609 and T2DM in the Karachi-based Sindhi population. Our results advocate that T2DM risk is especially raised in those with the risk A-allele of *FTO* variant rs9939609. The outcomes recommended that specific variants of the *FTO* gene could be used as a source to classify individuals who are more prone to the development of T2DM certainly in Asian individuals. In addition, this study also declares the *FTO* variant association with BMI and WC. However, further analysis with a larger sample size and confounding variables data, for instance, physical activity and diet intake, is needed to clarify the role of this variant as well as additional variants of the *FTO* gene on the predisposition to T2DM in Pakistani cohorts. It is a rising need to do more studies on T2DM susceptible genes with different polymorphisms to improve insight into the role of genes in predisposition to T2DM susceptibility. In future, this will generate a great target for drug discovery and development besides, the field of pharmacogenomics for clinical implementation.

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6. CONFLICT OF INTEREST

The authors declared that they have no competing interests.

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