Research Article

Common Hyperglycemia Susceptibility Loci in Metabolic Syndrome Patients from Karachi, Pakistan: A Cross Sectional Study

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Abstract

Background: More than 75 genetic susceptibility loci have been implicated with hyperglycemia. SNPs in genes linked with satiety, insulin sensitivity, BMR (Adiponectin, FTO, and MC4R), and inflammation and autoimmunity (TNF-a) have been shown to have a relationship with metabolic syndrome.

Objectives: To measure the frequency of ADIPOQ, FTO, MCR4, and TNF SNPs in metabolic syndrome patients and controls, and to assess the association of these with hyperglycemia and adiposity status.

Methods: A cross-sectional study was conducted. Baseline investigations including lipid profiles, blood glucose levels, and BMI calculation were conducted for n=113 Metabolic Syndrome and n=47 healthy controls. Tetra ARMS PCR and gel electrophoresis were conducted for Adiponectin rs266729, Adiponectin rs1501299, FTO rs9939609, MCR4 rs1297013, and TNF rs1800629. Statistical analysis was performed using SPSS version 26.

Results: Difference was observed in lipid profiles (including LDL, triglycerides, and cholesterol but not HDL), body fat percentage (p= 0.034), and both fasting (p=0.000) and random (p=0.000) blood glucose levels among metabolic syndrome patients versus controls. Adiponectin rs1501299, FTO rs9939609, and MCR4 rs1297013 showed genotype frequency differences between groups. Spearman's correlation was applied to test any association of risk allele with study variables and MCR4 rs1297013 showed an independent association with Fasting Blood Glucose Level (r=0.238; p=0.024) irrespective of age, weight, or gender.

Conclusion: MCR4 rs1297013 polymorphism is an independent risk factor leading to hyperglycemia in the study population, however, no other SNPs were identified to carry a significant risk. Large-scale genome-wide studies are required to identify the unique set of risk genes for the Pakistani population.

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Introduction

The prevalence of diabetes has doubled over the past 3 decades, with an estimated 439 million adults expected to be affected by 2030. Global trends are worrisome even for the developing world, with the greatest relative increase over the next two decades expected in the same low-middle income countries (21.1%), in comparison to high (12.2%) and low-income countries (11.9%).[1] Pakistan has the highest age-standardized prevalence of type 2 diabetes in adults in South Asia, with approximately 9.8% of the total adult popu-

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lation affected.[2,3]

The genetic component in the pathophysiology of diabetes is significant, with higher concordance rates of type 2 diabetes observed in monozygotic twins compared to dizygotic twins. Since the risk of hyperglycemia and Type 2 DM is polygenic, around 75 susceptibility loci have been discovered and implicated via genome-wide association studies to date.[4] This includes polymorphism in candidate genes like TCF7L2 (transcription factor 7-like 2), which has the strongest association with Type 2 DM amongst all identified loci, KCNJ11, IRS1, Adiponectin, MCR4 etc.[4,5] Adiponectin, coded by the Adipo Q gene, has been shown to have an insulin-sensitizing effect on cells and is involved in maintaining blood glucose levels. Adiponectin concentration and the risk of developing insulin resistance can be linked to single nucleotide polymorphisms in ADIPOQ. It also increases fatty acid oxidation and lowers serum triglyceride levels; therefore decreased levels of adiponectin are often reported in obesity.[6] It has been established that the concentration of Adiponectin in blood and therefore the risk of developing insulin resistance, further leading to hyperglycemia and Type 2 DM sequelae can be strongly linked to single nucleotide polymorphisms (SNPs) in ADIPOQ, the gene which codes for Adiponectin.[7-9] The most common single nucleotide polymorphism (SNP) of this gene is rs266729 (11,377C>G), it is located in the proximal promoter region of the ADIPOQ gene.[10] Carriers of G allele in the gene polymorphism rs 266729 constitute a higher risk factor for the development of MetS. Various studies have shown a strong association between the Fat Mass and Obesity (FTO) gene and both obesity and Type 2 DM. The FTO gene has several SNPs, including rs7202116, rs1421085, and rs9939609. The latter is most closely related to Type 2 DM in the Pakistani population. The A allele of FTO increases the risk of Myocardial Infarction in type 2 DM patients, independent of dyslipidemia and BMI. FTO significantly expresses itself in the Hypothalamus, where it controls the Basic Metabolic Rate. Overexpression of FTO has been linked to increased levels of ghrelin and increased adiposity, and it is believed to increase food intake without affecting energy expenditure. Therefore, analyzing the FTO rs9939609 polymorphism in populations with normal and diabetic glycemia could provide valuable insights into the predisposition of diabetes in a population.[11] Melanocortin-4-receptor (MC4R) gene is expressed in the hypothalamus and is a G-coupled receptor that controls the energy balance of the body. The rs12970134 has been associated with appetite, insulin resistance, lipid metabolism, obesity, and T2DM.[12] Tumor necrosis factor-alpha (TNF alpha) is an inflammatory cytokine released by mononuclear cells. The most widely studied polymorphisms are in the promoter region of the gene in positions -308 and -238, also known as rs1800629 and rs361525 respectively. For both types of diabetes, the TNF-alpha 308 rs1800629 has been more widely associated. Interestingly, previous studies have shown a correlation between G allele and diabetes mellitus.[13,14] But other conflicting results have also been obtained from India, where the GG genotype has not suggested any significant role in hyperglycemia.[15]

In Pakistan, obesity and type 2 diabetes mellitus are major public health concerns, with genetic factors believed to play a role in addition to lifestyle and environmental factors. Therefore, this study aimed to perform genetic mapping of metabolically healthy versus unhealthy adults via Tetra Arms PCR to identify gene mutations that may predispose to an early onset of type 2 diabetes. Thus, this study aimed to measure the Adiponectin rs1501299 (G/T), Adiponectin rs266729 (C/G), FTO rs9939609 (A/T), MCR4 rs12970134 (G/A), TNF 308 (G/A) polymorphisms by Tetra arms PCR.

Methods

This cross-sectional study was conducted on one hundred and sixty subjects at Aga Khan University from September 19 to October 21, 2022. The research volunteers were recruited from the waiting areas of the outpatient department. The research was approved by the Aga Khan University Ethics Review Committee (ERC number 2022-1309-21306). The study subjects from both genders, between the ages of 18-40 years were included in the study. While subjects with any chronic cardiometabolic illness or infectious diseases such as HIV, HBV, HCV or on anti-inflammatory medication, or pregnant or unfit for blood testing were excluded from the study.

The sample size was calculated using the Open-Epi website,[16] where confidence level of 95%, power of 90%, a prevalence of metabolic syndrome as 8% was taken according to previously published data sources.[17]

The minimum sample size calculated for this research was 160. Subjects were classified based on the Harmonized criteria[18] for metabolic syndrome into two groups. a) Metabolic Syndrome (n=90) and b) Healthy Controls (n=70)

After taking informed consent, age, weight, body mass index [South Asian Criteria as per WHO recommended i.e., between BMI of 18.5 to 22.9 kg/m^2 (normal weight) and above 23kg/m^2 (overweight/ obese), waist circumference and blood pressure were recorded for each participant. Ten millilitres of blood were collected after an overnight fast of 10 hours. Serum samples were tested for fasting lipid profile, fasting blood glucose levels. Buffy coat was used for DNA isolation using Qiagen DNeasy blood and tissue ki (#59824). Extracted DNA was amplified by the Polymerase chain reaction (PCR). The gene polymorphisms for Adiponectin rs266729, Adiponectin rs1501299, FTO rs9939609, MCR4 rs1297013 and TNF rs1800629 were measured by Tetra amplification refractory mutation system-polymerase chain reaction (T-ARMS-PCR) method. The PCR conditions were optimized by using a gradient thermal cycler (Eppendorf). The T-ARMS-PCR method employs two external primers, forward outer (FO) and reverse outer (RO) producing a control band and two allele-specific internal primers (RI and FI). This method simultaneously amplifies both alleles in one single-PCR tube. GoTaq G2 Hot Start Green Master Mix (M7422) was used for the PCR reaction. One reaction volume (10µl) included 3µl of GoTaq G2 Hot Start Green Master Mix, 1µl of water molecular biology grade, 1.5 µl each of outer and inner, forward, and reverse primers and 1µl of template DNA. Initial denaturation was done on 94°C for 10 minutes and an additional 30 seconds. Annealing of primers to template DNA was done on 65.7°C, 61.4°C, 66.2°C, 58.8°C and 68.9 for Adiponectin rs266729, Adiponectin rs1501299, FTO rs9939609, MCR4 rs1297013 and TNF respectively for 30 seconds. Initial extension was done on 72°C for 30 seconds followed by a final extension of 72°C for 10 minutes. PCR products were kept on 4°C until visualization on gel. Products were analyzed on 2% agarose gel by placing the gel in the gel documentation system (Figure 1). Data was analyzed by using SPSS version 23. Quantitative variables were expressed as mean ± standard deviation. The Mann Whitney U test, chi square or Fischer exact test was applied wherever applicable. The Spearman ranks correlation was applied to test the association of gene polymorphisms with metabolic syndrome characteristics. In all instances, a p value of <0.05 was considered significant.

Results

The detailed results are shown in tables 1-3. Briefly, the mean age for MetS group was 34 years versus 25 years for controls. Apart from serum HDL levels and diastolic blood pressures, significant differences were observed in all baseline examinations and laboratory findings in healthy controls and metabolic syndrome patients (p<0.05) (Table-1).

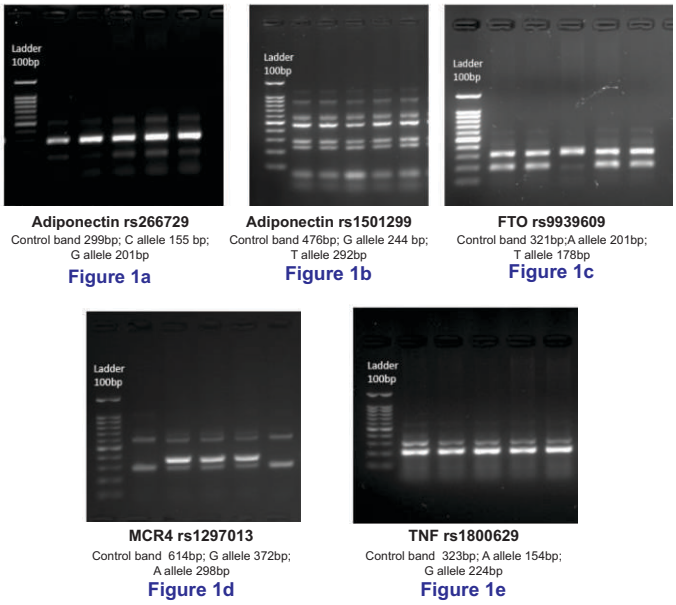

Figure 1a, b,c,d,e: *Gel Images of Tetra Arms PCR*

Significant differences were observed between obtained genotype frequencies for adiponectin rs1501299 (p= 0.038), FTO rs9939609 (p= 0.02), and MCR4 rs1297013 (p=0.001) when comparing the MetS group and controls (Table-2).

Significant correlations were observed between polymorphism in genetic loci Adiponectin rs1501299, FTO rs9939609, and MCR4 rs1297013, with body fat %, triglyceride, and fasting blood glucose levels respectively. However, once adjusted for age, gender, and weight only one correlation remained significant (MCR4 rs1297013 with Fasting Blood Glucose Level, P= 0.024) (Table-3).

Table 1: *Descriptive Statistics of the study Participants*

	Metabolic Syndrome n=90 Mean ± SEM	Healthy Control n=70 Mean ± SEM	P value
Age (Year)	34.03 ± 1.43	25.61 ± 0.77	0.001
Weight (Kg)	73.01 ± 1.82	64.91 ± 1.90	0.023
Body Fat (%)	29.03 ± 1.05	21.45 ± 1.11	0.034
Systolic Blood Pressure (mmHg)	121.810 ± 1.43	122.05 ± 1.25	0.034
Diastolic Blood Pressure (mmHg)	75.94 ± 1.61	70.88 ± 1.48	0.086
Waist Circumference (cm)	88.83 ± 2.14	85.68 ± 2.10	0.000
Cholesterol (mg/dl)	157.27 ± 6.39	146.35 ± 5.16	0.002
Triglycerides (mg/dl)	157.569±9.10	114.17 ± 7.66	0.007
Low Density Lipoprotein (mg/dl)	69.50 ± 5.98	38.88 ± 1.80	0.000
High Density Lipoprotein (mg/dl)	38.37 ± 1.45	52.62 ± 4.49	0.085
Fasting Blood Glucose (mg/dl)	127.77±10.90	81.47 ± 2.02	0.000
Random Blood Glucose (mg/dl)	174.15 ± 7.53	135.29 ± 1.63	0.000

Data presented as Mean± SEM. P value significance <0.05 Group comparison performed by T test

Table 2: *Chi square or Fischer Exact test for Gene/Allele Frequency*

Genes of interest		Metabolic Syndrome n=90	Healthy Control n=70	P value
		Genotype Frequency	Genotype Frequency	
Adiponectin rs266729	CC	90	70	>0.05
	CG	00	00	
	GG	00	00	
Adiponectin rs1501299	GG	07	00	0.038
	GT*	30	63	
	TT*	53	07	
FTO rs9939609	AA*	85	58	0.002
	TT*	02	14	
	AT	03	02	
MCR4 rs1297013	GG*	61	28	0.001
	GA*	17	34	
	AA	11	08	
TNF rs1800629	GG	90	70	>0.05
	AG	00	00	
	AA	00	00	

Genotype frequency is shown as absolute count. Chi Square Statistics were applied for comparing groups.

Table 3: *Correlation of genes of interest with Body Fat, Triglycerides and Blood Glucose Levels*

	Spearman's Correlation 'r'	P value	Adjusted for Age, Gender and Weight	P value
Adiponectin rs1501299 With Body Fat%	0.206	0.048	0.155	0.143
FTO rs9939609 with Triglycerides	-0.206	0.049	-0.155	0.415
MCR4 rs1297013 with Fasting Blood Glucose Level	0.227	0.030	0.238	0.024

Spearman's correlation was applied to test any association of risk allele with study variables followed by adjusted correlation for age, gender, and weight.

Discussion

Clinical diagnosis of MetS is complicated due to the heterogeneity of its presentation, and genetic factors, including over a hundred loci, have been linked to hyperglycemia, further complicating disease pathogenesis and prediction of risk.[18] The current study found significant differences in obtained genotype frequencies for adiponectin rs1501299 (p= 0.038), FTO rs9939609 (p=0.02), and MCR4 rs1297013 (p=0.001) when comparing the MetS group and controls. The greatest proportion (53%) of MetS patients had the TT genotype for adiponectin rs1501299, which was associated with body fat %. However, when adjusted for age, gender, and weight, the association no longer remained significant. A similar study of the Jordanian population measuring the same ADIPOQ gene SNP found its prediabetic subjects to have significantly higher glucose levels but significantly lower levels of serum adiponectin. At a genetic level, the study found the GT and TT genotype to increase the risk of prediabetes much more than the GG genotype.[19] This is like findings from our study where the greatest proportion of MetS patients had TT and then GT, in that order, while none of the MetS patients had the GG genotype. This highlights the association of the TT and GT genotype with hyperglycemic states. A study from Korea has further stressed the role of the T allele in particular, which has been found to cause arterial stiffness,[20] and thus may be associated with the microvascular complications occurring in hyperglycemic conditions of primarily diabetes and metabolic syndrome.

The FTO gene has also been linked to obesity risk, with

the homozygous AA genotype associated with a 1.67 times higher risk of obesity.[21-23] Previous studies from Turkey and Europe have noted similar frequencies, where around 19% of the participants had the AA genotype for the obesity risk allele, 42.5% of them had the AT genotype, and 38.5% of them had the wild-type TT genotype.[24] These results agree with our findings of 85% of the MetS group having the AA genotype and a significant difference being identified in comparison to the healthy group. In addition, a couple of studies from Pakistan found a strong association of the A allele in rs9939609 with type 2 diabetes but the SNP results were not statistically significant in correlation with obesity and BMI, similar to statistical findings in our study.[25,26] However, one study reported that the T allele was found to be associated with the risk of developing type 2 diabetes mellitus in Karachi.[27] We found a correlation with triglyceride levels which is actually contrary to previous reports that there is no association of FTO gene polymorphisms with triglycerides[28] but remarkably, an isolated laboratory study has found that an adipocyte-specific deletion of FTO can alter triglyceride metabolism.[29] Thereby a hypothesis for future studies could be that a gene polymorphism that reduces FTO activity could increase triglyceride levels, hence cause dyslipidemia and contributing to metabolic syndrome.

No statistically significant outcomes were obtained for TNF rs1800629 and Adiponectin rs266729 in our study. Contrarily in the past TNF has been heavily implicated in metabolic syndrome, studies have found TNF's association with insulin resistance and thus hyperglycemia and systematic analysis have even suggested its role as a biomarker to predict MetS.[13-15] Surprisingly, closer to our population group, a North Indian study found the AA genotype of TNF rs1800629 to be associated with T2DM,[30] while our study suggests the rs1800629 G>A to have a rather protective role considering 90% of the metabolic syndrome group had the GG genotype and a conversion of G to A could have prevented this occurrence. Similarly, for Adiponectin rs266729, only the CC genotype was found in our study. However, this could also allude to the idea that the A allele for TNF and G allele for adiponectin rs266729 is either absent or rare in the Pakistani population as our study failed to pick on any samples with these alleles. For further conclusive evidence, genome-wide association studies with a Pakistani sample size are needed.

When the results of our study were adjusted for age, gender, and weight only one correlation remained significant which was the MCR4 rs1297013 with fasting blood glucose, thus exhibiting that this polymorphism if present may be the direct cause of higher levels of glucose in the blood. MCR4 rs12970134 has however been significantly associated with body mass index in PCOS women in a past study with the A allele being correlated with a higher BMI and the consequent insulin resistance induced hyperglycemia which occurs in PCOS.[31] In contrast, a study from China found rs1297013 to be a low-frequency SNP in the Northern Han population and rs2331841 was found to be more closely related to obesity and its linked metabolic disorders.[32] A prospective study conducted on 1832 individuals from the Chinese Han population also established an association of AA allele of MC4R gene with T2DM.[33] A meta-analysis by Xi et al. concluded that these 2 SNPs were associated with T2DM after adjusting for BMI.[34] Thus, suggesting that different variants of the Melanocortin 4 Receptor gene can affect either the insulin function, metabolism, and/or food intake. Consequently, MCR4 rs12970143 may be in low frequencies outside Pakistan, but our study from Pakistan has found significant GG genotypes in metabolic syndrome patients, thus this may be a genetic feature of the Pakistani population for the MCR4 gene. Secondly, another Pakistani study tested for metabolite profile of the MCR4 gene and reported that it alters the fatty acid pathway.[35] Similarly, an Indian study has also reported an indirect association of MCR4 with obesity and hyperglycemia.[36] This may suggest that MCR4 SNP in Pakistan and Indian subcontinent is interconnected with altered metabolic pathways affecting various states of hyperglycemia. Hence our results show that specific genes are very likely of being associated with different metabolic states and thus in accordance with previously advised approaches could be used as a nutrigenomic tool for modifying interventions to reduce the risk of MetS.

Our study was limited by the small sample size, and being conducted in one center study, yet it shows a trend that the MCR4 rs1297013 polymorphism may be directly linked to hyperglycemia in the Pakistani population. However, its association with body mass index and metabolic disorders may vary in different populations. Therefore, further studies need to be conducted to estab-

lish the exact role of this SNP in different populations. Nevertheless, this study highlights the importance of understanding the role of specific genes in different metabolic states and the potential for using nutrigenomic approaches to modify interventions and reduce the risk of metabolic syndrome. Understanding the mechanisms underlying the genetic basis of MetS is essential for developing effective prevention and treatment strategies. In this regard, the role of different variants of the Melanocortin 4 Receptor gene in insulin function, metabolism, and food intake needs to be further elucidated.

Conclusion

MCR4 rs1297013 polymorphism is an independent risk factor leading to hyperglycemia in the study population. Contrary to available literature from the Caucasian population, common SNP's do not have a major role in the development of obesity or metabolic syndrome in a subgroup of the Pakistani population. A large-scale genome-wide population-specific studies are required to identify the unique set of genes for our population.

Ethical Approval: The Ethics Review Committee of The Aga Khan University approved the study vide letter number: Re 2022-1309-21306.

Conflict of Interest: The authors declare no conflict of interest.

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Authors' Contribution:

SA: Acquisition of data, drafting of article, final approval

HAI: Acquisition of data, drafting of article, final approval

SG: Acquisition of data, drafting of article, final approval

TT: Acquisition of data, drafting of article, final approval

VV: Acquisition of data, drafting of article, final approval

SF: Conception & design, analysis & interpretation of data, final approval

SSF: Conception & design, analysis & interpretation of data, drafting of article, critical revision for important intellectual content, final approval

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