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Association of Auto-Antigen SLC30A8 Gene Variants with Type 1 Diabetes Mellitus

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Abstract: Type 1 Diabetes Mellitus (T1DM), is a genetic disease, the prevalence of which is increasing due to delays in the diagnosis because of the absence of standard antibodies. Therefore, there is a need for a molecular variant that can help in early diagnosis in these patients. The polymorphism in the SLC30A8 gene can identify the people at risk of T1DM. However, there is a controversy in the association of the SLC30A8 gene in the pathogenesis of T1DM and a dearth of data in our part of the world. Therefore, we aimed to determine the most frequent autoantigen SLC30A8 genotype and its association with T1DM in the Pakistani population. This case-control study was carried out at Ziauddin Medical University (ZMU) jointly with Baqai Institute of Diabetology and Endocrinology (Baqai Medical University) from June to October 2019. A total of 50 subjects were enrolled (25 cases and 25 controls) in the study. Cases included 25 diagnosed patients of T1DM meeting American Diabetes Association (ADA) new criteria and controls were their first-degree relatives. Blood was drawn, DNA was extracted and amplified through PCR. The RFLP of PCR product was done using a restriction enzyme, Alu I for genotyping. The most frequently observed genotype was CC among cases as well as controls. The CC genotype of rs13266634 was not found significantly associated with T1DM (p=0.08) but the OR was 2.7; CI=0.86 -9.00. Similarly other genotypes were also not found statistically significant. However, HbA_{1C} and Fasting Blood Sugar (FBS) were found statistically significant (p=0.001, p=0.000) in T1DM patients compared to controls. The autoantigenic variants of SLC30A8 of rs13266634 were not found statistically significant with T1DM. The role of this variant as a susceptibility gene in T1DM development should be further confirmed by carrying out studies with a larger sample size.

Keywords: SLC30A8, Zinc transporter 8, Diabetes Mellitus, Type 1, variants.

自身抗原SLC30A8基因变异与1型糖尿病的关联

摘要：1型糖尿病是一种遗传性疾病，由于缺乏标准抗体而导致诊断延迟，其患病率正在增加。因此，需要一种可以帮助这些患者早期诊断的分子变体。SLC30A8基因的多态性可以识别有患1型糖尿病风险的人。然而，SLC30A8基因在1型糖尿病发病机制中的关联存在争议，并且在我们这个地区缺乏数据。因此，我们旨在确定巴基斯坦人群中最常见的自身抗原SLC30A8 基因型及其与 1 型糖尿病的关联。这项病例对照研究于 2019 年 6 月至 2019 年 10 月在齐奥丁医科大学与八开糖尿病与内分泌研究所 (八开医科大学) 联合进行。共有 50 名受试者（25 名病例和 25 名对照）参加了该研究。学习。病例包括 25 名确诊的 1

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型糖尿病患者，符合美国糖尿病协会的新标准，对照组是他们的一级亲属。抽血，提取脱氧核糖核酸并通过聚合酶链反应扩增。聚合酶链反应产物的限制性片段长度多态性使用限制酶铝1 进行基因分型。在病例和对照中最常观察到的基因型是 CC。未发现 rs13266634 的 CC 基因型与 1 型糖尿病显着相关（p=0.08），但优势比为 2.7；置信区间= 0.86 - 9.00。类似地，其他基因型也没有发现具有统计学意义。然而，与对照组相比，1型糖尿病患者的糖化血红蛋白和空腹血糖 具有统计学意义 (p=0.001, p=0.000)。 rs13266634 的 SLC30A8的自身抗原变异体在1型糖尿病中未发现具有统计学意义。这种变异作为易感基因在 1 型糖尿病发展中的作用应通过开展更大样本量的研究来进一步证实。

关键词：SLC30A8，锌转运蛋白 8，糖尿病，1 型，变体。

1. Introduction

Type 1 Diabetes Mellitus (T1DM), a serious organ-specific condition, is caused by autoimmune destruction of pancreatic β cells that secrete insulin, causing hyperglycemia disturbing the glucose homeostasis. It is usually diagnosed during childhood and adolescence but can also appear in the subsequent years of life [1,2]. This type of diabetes accounts for 5-10% of all diabetic cases and its prevalence is expected to increase 3% per year [3]. Subjects having multiple first-degree TIDM relatives are at increased risk of developing the disease [4]. T1DM clusters in families are reinforced by several environmental and genetic factors that contribute to the progress of T1DM clinical characteristics among different populations. Certain environmental factors like viral agents (causing lymphopenia) and dietary factors (exposure to gluten, consumption of cow's milk, vitamin D deficiency) may also participate in the β-cell autoimmunity [2,5]. Coxsackievirus B, rubella, cytomegalovirus, rhinovirus, and mumps have been recognized as a causative factors in certain T1DM cases [6,7]. The actual cause of T1DM is unknown, although it is observed to be a cell-mediated disease caused by immune system dysfunction, which results in a loss of β-cell antigen tolerance and harmful lymphocytic infiltration of the islets. As a result, deficiency of insulin compromises the homeostasis of glucose leading to clinical hyperglycemia [8].

Zinc transporter-8 (ZnT8), insulin, Glutamic acid decarboxylase (GAD), and Tyrosine phosphatase-related molecules-2 (IA-2) have been recognized as islet cell autoantigens. The zinc transporter ZnT8 is a six-domain transmembrane protein that facilitates the transport of zinc to the outside of the cell or into the lumen of intracellular organelles from the cytosol [9]. They are required for the storage, secretion, structural stabilization, and action of insulin.

The antigens, ZnT8, are frequently exposed during the glucose-stimulated exocytosis of insulin. In genetically susceptible individuals, this frequent exposure can stimulate and accelerate auto-antibody production against ZnT8 antigens [1]. ZnT8A has been identified to be frequent in children with new-onset T1DM in previous research, and it may be a predictor of disease risk [10,11].

ZnT8, a 369 amino acids protein, is located on chromosome 8 at position 24.11 on the q arm and is encoded by the SLC30A8 gene. A common single nucleotide polymorphism (SNP), rs13266634 SLC30A8 (C/T polymorphism) encodes either tryptophan (W) or arginine (R) by the T allele and C allele at aa325 of ZnT8. It is suggested that it may be important in T1DM autoimmunity [9].

This study aimed to explore the association of polymorphic variants of SLC30A8 with T1DM in the Pakistani population.

2. Methods

2.1. Study Design

It was a case-control study conducted after the approval of the Ziauddin Ethics Committee (ZEC). This study was carried out at Ziauddin University in collaboration with Baqai Medical University (BMU)-Baqai Institute of Diabetology and Endocrinology (BIDE). Using a convenient sampling technique from June to October 2019, a total of 50 subjects were enrolled in our study from the OPD of BIDE-BMU.

2.2. Participants

Diagnosed cases of T1DM patients (n=25) meeting the American Diabetes Association (ADA) criteria were taken as cases and their first-degree relatives (n=25) without any autoimmune diseases were taken as controls.

2.3. Analysis of Gene Variants

Whole blood was taken from all the cases and controls; the QIAamp blood mini kit was used for the

extraction of DNA from whole blood according to the manufacturer's protocol. The primer sequences listed below were used to amplify DNA:

Forward primer:
5'-GGACAGAAAGAGTTCCCATAGCG-3';
Reverse primer:
5'-ATAGCAGCATGTTTGAAGGTGGC-3'.

The protocol for PCR was 10 mins at 95°C, followed by denaturation, annealing, and elongation performed at 95°C for the 30s, 59°C for 30s, and 72°C for 30s, respectively, with a final elongation of 7 minutes.

After amplification by PCR of samples, all the individuals were genotyped by Restriction Fragments Length Polymorphism (RFLP) for rs13266634 C/T polymorphic variations of the SLC30A8 gene.

The PCR product was seen on a 2.5 percent agarose gel after being incubated for 16 hours at 37°C with the restriction enzyme Alu I (Thermo Scientific, USA). Two bands of 56bp and 125bp were detected when the homozygous wild-type CC genotype was present. 3 fragments at 56bp, 125bp, and 181 bp were detected as Heterozygous CT genotypes. While the undigested homozygous TT genotype was detected as 181 bp.

2.4. Statistical Analysis

The Statistical Package for the Social Sciences (SPSS) version 20.0 was used to conduct the statistical analysis. The numerical variables (age, FBS, BMI, HbA1C) were represented as mean and standard deviation. Gender, educational status, ethnicity, marital status, alleles, and genotypes were expressed as frequencies and percentages for categorical variables. Statistical significance was defined as a p-value of less than 0.05.

The association between genotypic variations and cases/controls was determined using the Chi-square test. The relationship of genotypes with cases and controls was determined using the odds ratio. The analysis of variance (ANOVA) was used to determine the differences in variables between the genotypic groups of cases and controls, followed by Post Hoc Tukey's analysis.

3. Results

3.1. Baseline Characteristics of the Study Population

There was a male predominance of 14 (56%) among the cases while 11 (44%) were females with a mean age of 20.72±6.34 years. Among the control group, there were 15 (60%) females and 10 (40%) males with a mean age of 41.12±11.20 years.

Table 1 lists the general characteristics of the subjects.

Table 1 General characteristics of the study population

Variables	Cases (Mean±SD)	Controls (Mean±SD)	p-value
Age (years)	20.72±6.34	41.12±11.20	0.000*
Age at diagnosis (years)	13.32±5.90	-	-
Disease duration (years)	7.74±5.85	-	-
Diastolic BP (mm/Hg)	72.6±12.00	82.05±10.70	0.009*
Systolic BP (mm/Hg)	106.80±13.54	117.50±13.71	0.012*
BMI (kg/m^2)	21.67±3.56	27.19±5.81	0.000*
FBS (mg/dl)	142.64±74.46	87.72±7.80	0.001*
HbA_{1C} (mmol/mol)	9.23±2.41	4.93±0.58	0.000*

3.2. Frequency of Variants

Among the subjects, the frequency of the C and A allele was determined to be 71 (71%) and 29 (29%) respectively. The frequency was 41 (82%) and 9 (18%) for C and T alleles respectively for cases while in controls, it was found to be 30 (60%) and 20 (40%) for C and T alleles respectively. Table 2 represents the genotypic (polymorphic) variants.

Table 2 Genotypic frequencies and their association with Type 1 Diabetes Mellitus

Genotypic variants	Cases n(%)	Controls n(%)	95% C.I.		O.R.	p-value
			Upper	Lower		
CC	18 (72)	12 (48)	0.86	9.00	2.7	0.08
CT	5 (20)	6 (24)	0.20	3.03	0.79	0.73
TT	2 (8)	7 (28)	0.04	1.20	0.22	0.08

OR= Odd's ratio, CI =Confidence interval, n = number of samples

3.3. Association of Variants

Despite the highest representation of CC genotype and high odds ratio among the cases, no statistical significance ($p<0.08$) was found with T1DM. PCR and RFLP of the polymorphic variants of SLC30A8 (rs13266634) are given in Fig. 1 and 2 respectively.

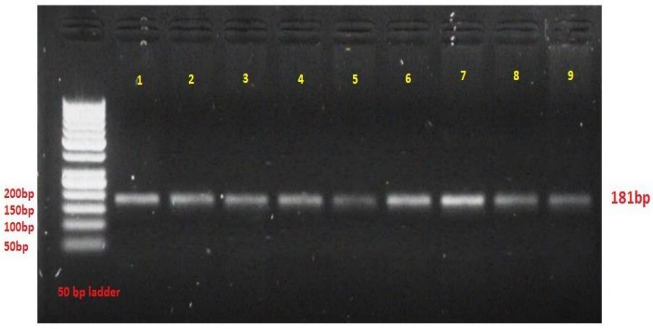

Fig. 1 Gel electrophoresis of the PCR product of SLC30A8 gene on a 2% agarose gel. Lane 1-9 shows the PCR product of 181bp against 50bp ladder

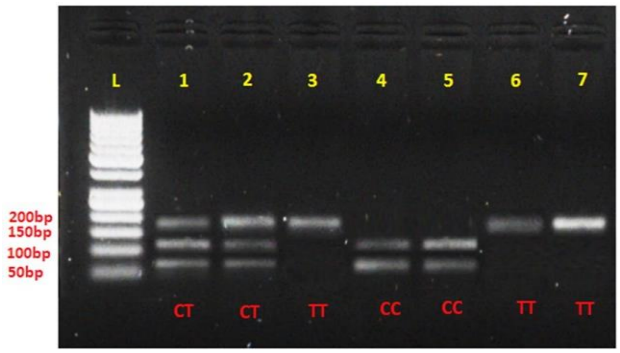

Fig. 2 Gel electrophoresis of SLC30A8 variant on 2.5% agarose gel. Lane 1,2: CT, Lane 3,6,7: TT, Lane 4,5: CC: 125,56 bp; CT: 181,125,56 bp; TT: 181bp

ANOVA was used to compare the SLC30A8 variants with variables in cases and controls. Among the cases, the study revealed no significant results except for age (p=0.008). TT for age exhibited a higher mean of 32.50±4.95, In Post- Hoc analysis, age differences between the CC and TT variants were statistically significant (p=0.007).

4. Discussion

Overall, the CC genotype was found to be the most frequent genotype displaying 72% in cases and 48% in controls. The OR of the CC genotype was 2.7 based on genotypic frequencies, indicating its possibility as a susceptibility gene, In studies conducted worldwide, CC genotype showed higher frequency as 63%,46.8%, and 43.1% in Indian, Danish and Japanese studies respectively [12-14]. Furthermore, in German research, the majority (65%) of T1DM patients less than 5 years of age had CC genotype compared to the remainder of the cases and controls [15]. Moreover, CC genotype was also frequently seen among the GDM patients who developed postpartum T1DM [16]. Because Pakistanis and Indians are genetically similar, these increased frequencies in various populations, particularly in the Indian population, imply the role of CC genotype in T1DM.

ZnT8 is highly expressed in pancreatic islet β-cells and has been identified as one of the four primary auto-antigens in T1DM patients. Zinc Transporter-8 Autoantibodies (ZnT8A) are found before the beginning of the disease, and rs13266634 C/T polymorphism variations of the SLC30A8 gene is known to be responsible for the autoimmune response [12]. ZnT8 is a six transmembrane domain protein that helps in the movement of Zn^{+2} in the intracellular compartment from the cytosol [17].

Researchers have identified SLC30A8 as a new antigenic target in patients in clinical studies, and its genetic variations have been linked to T1DM [18]. The results, however, are inconclusive. This study has added to the population statistics and also to the current literature.

In the current study, the mean age of diabetes onset was 13.32±5.90 years. Studies conducted across the world also displayed results ranging from 9 to 15 years [5,12,19]. In our study, male predominance was observed among the cases and similar results were found in an Indian [12] and Swedish study [20]. Female preponderance was reported in Brazilian research, which is likely due to the higher frequency of autoimmune diseases in women which is contrary to our results.

Among cases, the frequency of C and T alleles were found to be 41 (82%) and 9 (18%) respectively whereas, controls had allelic frequencies of 30 (60%) and 20 (40%) in the same order. The C allele was found to be more common in Type 1 diabetics (82%) than in controls in the current research (60%). In a German study, similar findings were found in children who developed diabetes before 5 years of age and it was also identified as a risk allele (82%) [15]. On the other hand, contrasting studies were found that showed no risk of C allele in T1DM development [12,21]. However, C allele rs13266634 of SLC30A8 was found to be associated with β – cell dysfunction but not with insulin resistance [21]. As a result, the differences in allele frequency were most likely due to genetic variances in the population.

In this study, a lack of association was found between the genetic variants of SLC30A8 rs13266634 and T1DM. An Indian researcher reinforced our findings by meta analyzing its results with other research [12]. Xu et al. also conducted another meta-analysis and came to the same conclusion [22]. Furthermore, Japanese research found that genetic variants of SLC30A8 don't alter the risk for T1DM even in younger individuals [23]. Contrasting results were found in some studies showing higher CC genotype of SLC30A8 variants of rs1326634 in early-onset T1DM [15,21]. Inadequate autoimmunity mediated β-cell dysfunction along with C/T polymorphism of SLC30A8 predisposes Type 2 Diabetes Mellitus development. C/T polymorphism is also significantly associated with HbA_{1C} and impaired glucose tolerance in T2DM [12,22,24]. Environmental variables like infections and diet may participate in the development of the disease by modifying the autoimmune response and SLC30A8 gene rs13266634 may not be the susceptibility gene of T1DM. A recent study suggests that the SLC30A8 rs2466293 gene's adjacent locus may enhance the risk of T1DM development in non-European lineages [5,19]. As noted previously in our research paper, ZnT8A may serve as an independent biomarker for the susceptibility of T1DM but not rs13266634 C/T genotypic variants.

When the variables were compared, it showed a higher mean of 32.50±4.95 years of age in the TT genotype of the case group, indicating that this genotype was frequently observed (p=0.008) in older T1DM patients. In the comparison of other variables

(age, FBS, BMI, HbA_{1c}), no significant observations have been found in both groups. No such data is obtainable from the literature regarding these variables. The only limitation of this study was the sample size which may limit the detection of associations and significant differences.

5 Conclusion

SLC30A8 genotypic variants were not found to be significantly associated with the development of T1DM. This information may aid in understanding the role of SLC30A8 gene polymorphism in patients with Type 1 Diabetes Mellitus providing baseline knowledge because this is the first study on the variants of SLC30A8 in the Pakistani population. The main limitations of the study were that the patients were not recruited at the time of T1DM diagnosis and the smaller sample size of the study. Screening of newborn babies with a positive history of T1DM showing CC genotype should be followed up for the development of T1DM. SLC30A8 genotypes should also be associated with ZnT8A variants in T1DM. To validate our findings regarding the role of gene polymorphism in T1DM, this study needs to be replicated with a bigger sample size along with further clinical, molecular level, and interventional investigations to elucidate the role of other genetic variants and to test it as a susceptibility gene in T1DM patient. Current bioinformatics tools should also be explored and used to diagnose new biomarkers in T1DM and rationale to identify the patients in the early phase of the disease and to avoid future complications.

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