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Original Article

Screening of Type 2 Diabetes Mellitus Patients of Khyber Pakhtunkhwa for SLC30A8 (rs13266634) Variant Associated with Disease Susceptibility

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ABSTRACT

Diabetes Mellitus (DM) is a pressing global health concern. The SLC30A8 rs13266634 variant, linked to Type-2 DM (T2DM) susceptibility, presents a unique research opportunity to understand genetic factors influencing disease outcomes and guide personalized treatment approaches. **Objective:** To evaluate the association of the SLC30A8 rs13266634 variant with Type 2 Diabetes Mellitus (T2DM) susceptibility among patients in Khyber Pakhtunkhwa, Pakistan. **Methods:** A case-control study design was employed involving 100 each T2DM patients and healthy controls. Demographic and clinical features were recorded. The SLC30A8 rs13266634 variant was genotyped using PCR-RFLP. Statistical analyses, including binary logistic regression, were conducted to determine the association between the variant and T2DM, adjusting for age, gender, family history, and lifestyle factors. **Results:** The study cohort comprised predominantly males (65% in cases, 75% in controls) with a mean age of 53±9 years. T2DM patients exhibited a higher prevalence of concurrent conditions such as high blood pressure compared to controls. Genotyping revealed a significant association of the rs13266634 variant allele with T2DM. Individuals carrying variant CT and TT genotype had a 2.12 times higher risk (95% CI: 1.16-4.12, P=0.025) of T2DM compared to that of wild CC type. This association remained significant upon adjustment for confounders, with an adjusted odds ratio of 2.890 (95% CI: 2.233-9.76, P=0.02) for CT carriers. **Conclusions:** The rs13266634 variant in the SLC30A8 is significantly associated with an increased risk of T2DM in the Khyber Pakhtunkhwa population.

INTRODUCTION

Diabetes Mellitus (DM) is a chronic metabolic disorder characterized by persistent hyperglycemia (high blood sugar levels), which results from defects in insulin secretion, insulin action, or both. The World Health Organization (WHO) and the International Diabetes Federation (IDF) have highlighted diabetes as a significant global health crisis. The disease is a leading cause of cardiovascular diseases, kidney failure, blindness, and lower limb amputation, making its management a critical public health challenge. Diabetes prevalence will climb to 5.4% by 2025, indicating both the disease's growing prevalence as well as the rising cost and severe danger to the public. According to the International Diabetes Federation, diabetes would affect 578 million people by 2030, an increase from the present predicted 3710 lakh cases [5]. Just 50% of diabetics know of their condition, and of those that are, 91 % have T2DM, whereas the remaining has type 1. National Diabetes Fact Sheet of 2011 states that, 8.3% of Native Americans, irrespective of their ages, have diabetes, with individuals of age 20 or older accounting for 11.3% of those affected. In 2010, around 215,000 persons below age 20 had diabetes, predominantly

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T1DM or T2DM, with those aged 65 and above making up 25% of the sum. Diabetes affects around 7 million Americans, 27% of whom are diabetic . In recent years there has been seen a significant and steady rise in diabetes prevalence in Pakistan, making it one of the most impacted countries worldwide by this chronic disease. The International Diabetes Federation reports that the rate of adults with diabetes in Pakistan increased from 11.77% in 2016 to 17.1% in 2019, and strikingly, it soared to 26.7% by 2022 . This equates to around 33 million people living with diabetes—a figure that underscores the pressing public health challenge facing Pakistan. Contributing factors include rapid urbanization, shifts in lifestyle, and dietary preferences for processed foods. Additionally, the contrast in diabetes prevalence between urban (15.1%) and rural areas (1.6%) highlights the influence of urban lifestyles on the occurrence of the disease. The escalating obesity rates, with 57.9% of the population experiencing generalized obesity and 73.1% facing central obesity, exacerbate the situation, paving the way for a surge in diabetes and prediabetes incidences .The presence of a disease within families indicates a genetic cause of diabetes. Diabetes risk increases as the number of sensitive genes in the family increases, especially if the disease runs in the family. People who have an immediate family relative with T2DM are likely to develop the disease themselves. A child's frequency of getting the disease gets higher by a factor of 40 if one of their parents has the disease, and by a factor of 70 if both of their parents have the disease .SLC30A8 is a member of the solute carrier family 30 (zinc transporter) located on chromosome 8. The gene is responsible for protein formation which then stores and secretes insulin and is only found in the islets of Langerhans. 2 zinc ions unite with and stabilize a hexagonal form of insulin, triggering insulin crystallization. This proposes that the gene might be involved in T2DM progression and the connection is confirmed by other studies–. This gene encodes a zinc efflux transporter, which accumulates zinc ions in vesicles. There are allelic variants of this gene that are associated with T2DM. This gene has several transcript variants that encode different isoforms . SLC30A8 is specifically expressed in both alpha and beta cells islet and has lower expression levels in the testes, and other body parts. This transporter is required for zinc to enter beta-cell insulin-secretory granules and then crystallize hexametric insulin .When glucose is stimulated, the insulin in secretory vesicles is ready for immediate release. Abnormalities in this process are thus expected to impact the acute insulin response to glucose. Rs13266634 is a SNP (single base change CT) that encodes a non-conservative amino acid change at position 325 (R325W) from arginine to tryptophan. The mechanism by which this variant (R allele) increases the risk of T2DM is likely due to insufficient insulin processing and secretion based on its function in pancreatic islets. A study of people with a family history of type 2 diabetes found that those with the RR genotype of rs13266634 had a lower first-phase insulin response to an intravenous glucose load during a frequently sampled IVGTT (FS-IVGTT) than those with the WW genotype. The rs13266634 C is replaced by T, and two 3'UTR variants (rs2466294 C/G and rs2466293 T/C) in SLC30A8 gene had been linked to T2DM. Other rs7002176, rs1995222, rs1995222, and rs16889462 are variants of the said gene– .The SLC30A8 rs13266634 variant's role in T2DM among the Khyber Pakhtunkhwa population in Pakistan has not been extensively studied, presenting a unique opportunity to fill this knowledge gap. Understanding the genetic factors that influencing drug responsiveness is crucial for optimizing treatment outcomes.

SLC30A8 rs13266634 variant is significantly associated with increased susceptibility to T2DM in the Khyber Pakhtunkhwa population of Pakistan. Our objective is to investigate the prevalence and impact of this genetic variant on T2DM; offering insights that could guide personalized treatment strategies. By focusing on the genetic factors influencing T2DM susceptibility, we aim to enhance our understanding of the disease's pathophysiology and support the development of tailored interventions that could improve patient outcomes and contribute to the field of personalized medicine.

METHODS

Study Duration and Data Collection Sites

The research was conducted from October 28, 2021, targeting the association of the SLC30A8 rs13266634 variant with T2DM in Khyber Pakhtunkhwa, Pakistan. Data were sourced from category D healthcare facilities in Peshawar. Ethical clearance was secured from the University of Peshawar's Department of Pharmacy, evidenced by clearance number 411/EC/F.LIFE/UOP-2021.*Subject Selection and Sample Collection:* The scope of the study research was calculated using the hypothesis of a 5% level of significance, 90% power, and a 4 odds ratio from Table 10 of the WHO's manual for sample size. The ethics committee of the Department of Pharmacy at the University of Peshawar has given clearance (411/EC/F.LIFE/UOP-2021), dated October 28, 2021, to proceed. A sum of 200 subjects and healthy population were chosen from category D setups in Peshawar, KP. Both research groups showed no hesitation in agreement of dully signed carefully constructed questionnaire form (annexure II) based on a number of T2DM-related factors such as family information, socio-economic conditions, ages, life style, clinical hallmarks, and early medical diagnosis. 3 mL of whole blood was drawn using a sterilized syringe . Inclusion criteria were identified T2DM patients

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with a relationship to family genetics tendency of any gender and age over 35 years. Exclusion criteria were insulin-dependent diabetic patients, T2DM patients who do not have a family history of the disease, pregnant diabetic women (gestational diabetes), T2DM patients under the age of 30 and Diabetics who simultaneously have a long-term concurrent condition such as HIV, end stage kidney disease etc.

DNA Extraction and PCR Amplification

Genomic DNA was extracted from peripheral blood samples using the WizPrep DNA extraction kit (WizPrep, No. W54100) according to the manufacturer's instructions. The SLC30A8 rs13266634 variant was genotyped using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) method. The PCR amplification was carried out using DreamTaq Green PCR Master Mix (2X, Thermo Fisher Scientific) with specific primers: forward primer 5'-GGACAGAAAGAGTTCCCATAGCG-3' and reverse primer 5'-ATAGCAGCATGTTTGAAGGTGGC-3'. The amplification conditions were as follows: initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 1 minute, with a final extension at 72°C for 10 minutes .

Restriction Digestion and Genotyping

The PCR products were digested with the ACIL restriction enzyme (specific for the rs13266634 SNP) at 37°C overnight. The digested fragments were then separated on a 2% agarose gel, stained with ethidium bromide, and visualized under UV light. The presence of the cut (variant) and uncut (wild type) alleles was determined based on the size of the restriction fragments: 429 bp for the uncut allele and 234 bp and 195 bp for the cut alleles.

Statistical Analysis

Data were analyzed using SPSS version 26. The association between the SLC30A8 rs13266634 variant and T2DM was evaluated using binary logistic regression to compute odds ratios (ORs) with 95% confidence intervals (CIs), adjusting for potential confounders including age, gender, family history, and lifestyle factors. A P-value of <0.05 was considered statistically significant.

RESULTS

Population vital statistics and Clinical features of Study Subjects

Our study involved 200 participants, equally divided between 100 individuals diagnosed with Type 2 Diabetes Mellitus (T2DM) and 100 healthy controls. The investigation sought to elucidate the age-related prevalence and characteristics of T2DM among the studied population. The mean age of our T2DM samples was 53± 09 (SD) years. Table 1-2, representing the clinical and demographic variables of the investigation's participants.

Table 1: Demographics Variable of Investigation's Participants.

Variables	Case (N)	Control (N)
Gender		
Male	67	79
Female	33	21
Subject Ages		
31-40	2	10
41-50	36	32
51-60	39	42
61-70	22	14
71-80	1	2

Table 2: Prevalence of Concurrent Medical Conditions Among Study Participants

S. No.	Diseases	Case (N)	Control (F)
1	Kidney Diseases	08	0
2	Cholesterolemia	03	02
3	High BP	31	12
4	Ophthalmic Injury	41	1
5	Cardiac Disorder	16	0
6	Hepatitis B Virus (HBV)	0	0
7	Hepatitis C Virus (HCV)	01	0
8	No Concurrent Conditions	0	85

SLC30A8

The investigation into the SLC30A8 variant involved a precise amplification process utilizing Polymerase Chain Reaction - Sequence-Specific Primer (PCR-SSP) technique. This method was meticulously executed under optimal conditions to ensure the accurate binding of primers, a critical step for the specificity of the amplification. Following the PCR, electrophoresis was conducted on the amplified products to analyze the variants. The SLC30A8 variant yielded an amplicon with a length of 429 base pairs (bp), necessitating the use of a 100 bp ladder during electrophoresis for accurate size determination. This process enabled the clear visualization and verification of the variant's presence in both the case and control groups. The outcomes of these analyses are detailed in Figures 1 and 2, illustrating the electrophoresis results for the case and control samples, respectively.

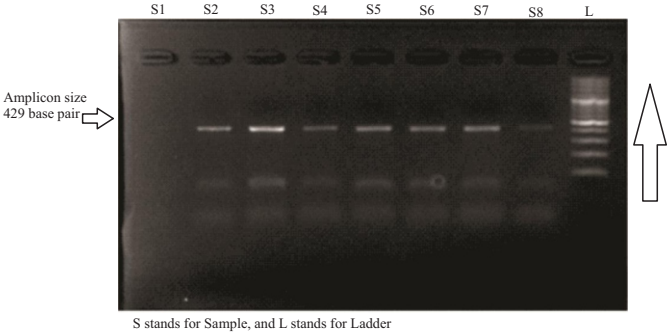

Figure 1: PCR Product (Case) Electropherograms for SLC30A8 Gene Using 100 BP Ladder

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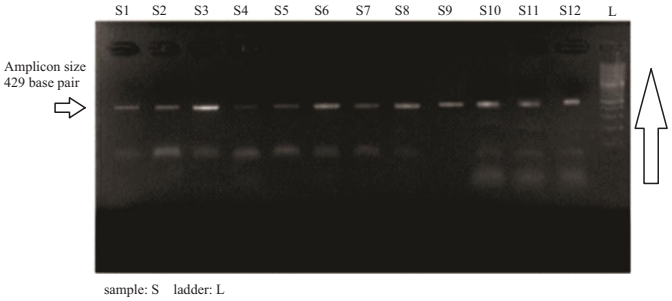

Figure 2: PCR Product (CONTROL) Electropherograms for SLC30A8 Gene Using 100 BP Ladder

Outcomes of the SLC30A08 Gene Following the Implementation of a Restriction Enzyme

The variation present in the SLC30A08 gene was identified through the utilization of a 2% agarose gel, a ladder consisting of DNA fragments 50 base pairs long, and the ACIL restriction enzyme. Upon subjecting the PCR product to enzymatic digestion at the specific sites, namely cytosine-cytosine, cytosine-thymine and thymine-thymine, fragment lengths of 429, 234, and 195 base pairs were obtained, respectively. Electropherograms depicting the genetic variations within the SLC30A08 gene for both the case and control groups are visually represented in figures 3, and 4. The data presenting the prevalence of these variations in the two groups is summarized in table 3, and a visual representation is provided in figure 5.

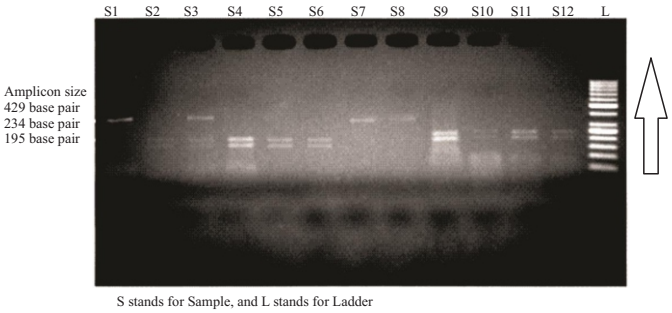

Figure 3: PCR Product (Case) Electropherograms for SLC30A8 Gene Using 50 BP Ladder

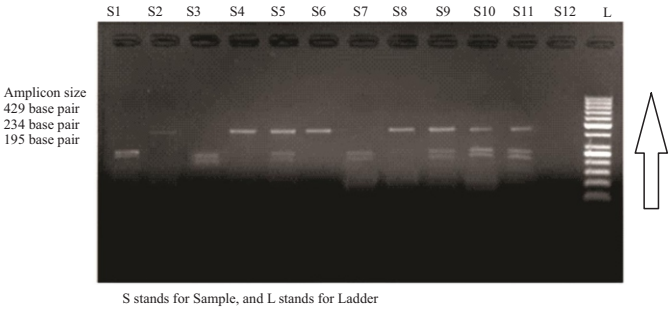

Figure 4: PCR product (Control) Electropherograms for SLC30A8 gene using 50 BP Ladder

In both control and diseased individuals, amplicons of 429 BP were observed, indicative of the presence of the uncut SLC30A8 rs13266634 allele, signifying the CC genotype. Additionally, amplicons of 235 BP and 194 BP, resulting from the enzymatic digestion of the PCR product, were present in both groups, representing the CT and TT genotypes, respectively. This pattern of amplicon sizes across both cohorts suggests the widespread presence of these genotypes within the studied population, underscoring the necessity of genotypic analysis in understanding the genetic predisposition to Type 2 Diabetes Mellitus (T2DM).Despite the minimal difference in genotype frequencies between the control and diseased groups, a significant association of the CT and TT genotypes with an increased risk of T2DM was identified. This conclusion was drawn not merely from the genotype frequencies themselves but from the statistical analysis that accounted for potential confounders. The binary logistic regression analysis, adjusting for age, gender, family history, and lifestyle factors, revealed that individuals carrying the CT or TT genotypes had a significantly higher risk of developing T2DM compared to those with the CC genotype. This analysis underscores the nuanced nature of genetic influences on disease predisposition, where even small differences in genotype frequencies, when considered within the broader context of genetic and environmental interactions, can elucidate significant associations with disease risk.

Genotype Determination

Genotyping of the SLC30A8 rs13266634 variant was conducted using the Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) technique. Post-PCR, the amplicons were subjected to digestion with the ACIL restriction enzyme, specifically recognizing the SNP site of rs13266634. The digestion process resulted in the generation of different fragment sizes, indicative of the CC, CT, and TT genotypes. Uncut alleles, representing the CC genotype, produced a single fragment of 429 BP. In contrast, the presence of the CT genotype was inferred from the production of both cut and uncut fragments, resulting in sizes of 234 BP and 195 BP, alongside the 429 BP fragment. The TT genotype was identified through the complete digestion of the amplicon into fragments of 234 BP and 195 BP, with no 429 BP fragment present. The digested products were resolved on a 2% agarose gel, stained with ethidium bromide, and visualized under UV light for genotype determination.

SLC30A8 rs13266634 variant Association with DM2

The demographic data indicated that the average age of both the control group (consisting of healthy individuals) and the group with Type 2 Diabetes (T2DM) patients was approximately 52 years with a standard deviation of 9.91, and the statistical significance (P-value) of this

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observation was 0.304. Among the T2DM patients, 46% fell within the age range of 40 to 60 years. The ratio of males to females was 65.35, and the greatest occurrence of T2DM was observed in Peshawar (53%), followed by Charsadda (13%) and Mardan (12%). The primary occupations among the patients were homemakers (34%) and workers (20%). Furthermore, a large majority of the patients (94%) had a family history of T2DM, and most of them (85%) did not engage in regular exercise. Notably, a significant difference was observed in the arrangement of both cases and controls concerning the SLC30A08 gene variation rs13266634. For individuals possessing the CC homozygous genotype, a significant association was observed in those with the CT variant, as demonstrated by an odds ratio of 2.12 and a 95% confidence interval ranging from 1.16 to 4.12 (P=0.025).Similarly, this relationship remained significant when the CC genotype was considered, showing a 2-fold association (odds ratio: 1.43, 95% confidence interval: 0.83-2.89, P=0.239). Importantly, when accounting for factors such as family history, age, socioeconomic status, lack of exercise, and dietary habits, the impact of these genetic variations remained significant (adjusted odds ratio: 2.890, 95% confidence interval: 2.233-9.76, P=0.02; and odds ratio: 1.85, 95% confidence interval: 1.193-3.570, P=0.021, respectively). Our research conclusively highlights a significant link between Type 2 Diabetes Mellitus (T2DM) in the population under study and the SLC30A8 gene variant (rs13266634). The allelic frequencies of the wild-type and the risk variant within the SLC30A8 gene are detailed in 3 and illustrated in Figure 3, facilitating a comprehensive comparison.

Table 3 : Frequency Of Allelic Variants

Geno type	Control N (%)	Case N (%)	Unadjusted OR (95% CI)	P-Value	Adjusted OR (95% CI)	P-Value
CC	56.9	61.3	Ref	Ref	Ref	0
CT	34.7	30.2	2.12 (1.16-4.12)	0.025	2.890 (2.233-9.76)	02
TT	5.4	8.5	1.43 (0.83-2.89)	0.239	1.85 (1.193-3.570)	12

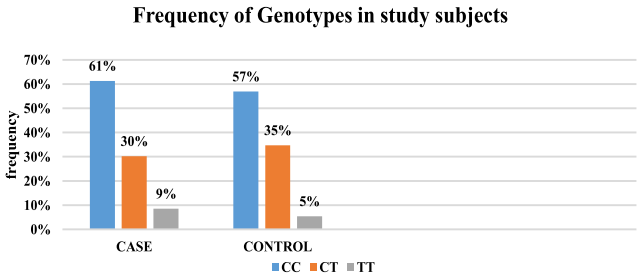

Figure 5: Occurrences and Ratio of Genotype CC, CT, and TT among Control and Case Participants

The CT genotype shows a statistically significant association with the outcome. Individuals with the CT genotype have 2.12 times higher odds of the outcome compared to those with the CC genotype. This association remains statistically significant even after adjusting for confounding factors, with an adjusted odds ratio of 2.890. The TT genotype does not show a statistically significant association with the outcome when considering the unadjusted odds ratio. However, after adjusting for confounding factors, the association becomes statistically significant, with an adjusted odds ratio of 1.85. It's important to note that p-values and odds ratios alone do not provide a complete understanding of the results. Context about the study design, the size of the sample, the quality of data collection, and the nature of the outcome and genetic variant being studied are all crucial for interpreting these findings accurately. Additionally, replication studies and further research are often needed to confirm and generalize these findings.

DISCUSSION

Our study revealed a significant association between the SLC30A8 rs13266634 variant and the susceptibility to Type 2 Diabetes Mellitus (T2DM) among the Khyber Pakhtunkhwa population in Pakistan. Individuals carrying the CT and TT genotypes of this variant exhibited a markedly increased risk of developing T2DM compared to those with the CC genotype. These findings align with the growing body of evidence suggesting a genetic predisposition to T2DM, influenced by specific allelic variations. Upon comparing our results with existing literature, the association between the rs13266634 variant and T2DM risk has been consistently reported across different populations. For instance, studies conducted within Asian and European cohorts have identified the rs13266634 variant as a significant risk factor for T2DM, underscoring the variant's potential as a universal marker for diabetes susceptibility. However, the degree of risk associated with the CT and TT genotypes varies, likely due to population-specific genetic backgrounds and environmental factors. Interestingly, while our findings corroborate the general consensus on the role of SLC30A8 rs13266634 in T2DM, the observed effect size in the Khyber Pakhtunkhwa population suggests a potentially stronger influence of this variant compared to reports from other regions. This difference could be attributed to unique genetic interactions or lifestyle factors prevalent in this specific demographic. Our study also contributes to the understanding of T2DM pathogenesis by emphasizing the importance of genetic screening in at-risk populations. Identifying individuals with a genetic predisposition to T2DM could facilitate early intervention and personalized management strategies, potentially mitigating the disease's impact. However, it is crucial to interpret these findings within the context of the study's limitations. The scope of our analysis was confined to a single genetic variant within a specific population, which may not fully capture the complex genetic

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architecture of T2DM. Future research should aim to explore additional variants and their interactions, as well as consider the role of environmental factors in modulating T2DM risk.

CONCLUSIONS

The present study indicated that the rs13266634 C allele in the SLC30A8 gene was associated with T2DM risk in the Khyber Pakhtunkhwa population. More studies are needed to confirm the association between rs13266634 and the said population. Our study robustly establishes the SLC30A08 gene variant (rs13266634) CT and TT variants, as a significant contributor to T2DM susceptibility within our examined population.

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Authors Contribution

Conceptualization: SSA, HB
Methodology: SSA, HB
Formal analysis: SSA, HB, HS, SA, MK, FK, KI, MR, ZK
Writing, review and editing: SSA, HB, HS, SA, MK, FK, KI, MR, ZK
All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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