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Association of SNP rs533864137 with Severe and Early Diabetic Nephropathy in the Pakistani Population

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ABSTRACT

Objective: To explore the association between SNP rs533864137 and diabetic nephropathy in a Pakistani cohort using tetra-primer amplification refractory mutation system-polymerase chain reaction (ARMS-PCR).

Study Design: Case-Control study.

Place and Duration of Study: Center for Research in Experimental and Applied Medicine (CREAM), within the Department of Biochemistry & Molecular Biology, Army Medical College, Rawalpindi, Pakistan from Nov 2020 to Aug 2024.

Methodology: A total of 213 participants, comprising 113 DN Patients and 100 Controls with diabetes but without nephropathy, were included. Genomic DNA was extracted from blood samples, biochemical analysis was performed to ascertain cases and Controls, and SNP rs533864137 was genotyped using ARMS-PCR. Statistical analyses evaluated the distribution of genotypes and alleles between the Groups and assessed the SNP's association with DN.

Results: Among Controls, 97(97%) individuals had the GG genotype, and 3(3%) had the GA genotype. In the DN Group, 100(99.1%) exhibited the GG genotype, while 1(0.9%) was homozygous for the AA genotype. No significant differences in genotype distribution were observed between Groups (p=0.310). Statistical analysis confirmed that rs533864137 was not significantly associated with DN (p>0.05).

Conclusion: SNP rs533864137 does not appear to be associated with DN in this population. These findings suggest that rs533864137 may not serve as a reliable genetic marker for assessing DN risk in the studied cohort.

Keywords: Diabetic Nephropathy, SNP rs533864137, Tetra Primer-ARMS-PCR, CBR1 Gene.

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INTRODUCTION

Diabetes mellitus is a major global healthcare challenge. One of the fastest-growing diseases in the world, with 536.6 million people (10.5% of the world population) living with diabetes in 2021, and this number is expected to rise to 783.2 million people (12.2%) by the year 2045, according to the International Diabetes Federation (IDF).[1] Diabetic nephropathy (DN) is a prevalent and severe complication of diabetes mellitus (DM), significantly contributing to morbidity and mortality among diabetic Patients worldwide.[1] Characterized by progressive kidney damage, DN often leads to end-stage renal disease (ESRD), necessitating dialysis or kidney transplantation.[1] Almost 25% of diabetics develop severe DN early in the course of the disease and progress rapidly to the end stage of renal failure within less than 10 years of diagnosis of diabetes.[2] Every fourth person in Pakistan is a diabetic, according to the second national diabetes survey of Pakistan (NDSP).[3] Recent studies highlight a growing diabetes crisis in Pakistan. As of 2023-2024, Pakistan has one of the highest diabetes prevalence rates globally, with around 30.8% of its population affected.

Diabetes mellitus is a complex, multifactorial disorder characterized by the intricate interplay of environmental, genetic, metabolic, sociocultural determinants and mediators of inflammation (including IL-1, MCP-1, MMP-9, CTGF, TNF-α, and TGF-β1).[4] Vascular damage is primarily attributed to glucose toxicity, which is mediated through various metabolic mechanisms. These include the synthesis of reactive oxygen species, the activation of the polyol pathway, the formation of advanced glycation end products (AGEs), and the activation of protein kinase C pathways.[5] The CBR1 gene is involved in methylglyoxal metabolism6. Carbonyl reductase-1 detoxifies methylglyoxal as an alternate pathway for MG metabolism.[6] It has been demonstrated that the CBR1 enzyme helps with a variety of medical disorders and lowers oxidative stress. Carbonyl compound scavengers like pyridoxamine, L-carnosine (β-alanyl-L-histidine), and Tenilseta, along with inhibitors of their synthesis such as Benfotiamine, have shown potential in mitigating carbonyl stress-

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associated disorders.[7] Dicarbonyl compounds, such as 3-deoxy fructose and 2-keto-3-deoxy gluconic acid, arise from the polyol pathway (Figure 1a), whereas glyoxal and methylglyoxal result from sugar glycoxidation,[8] (Figure 1b).

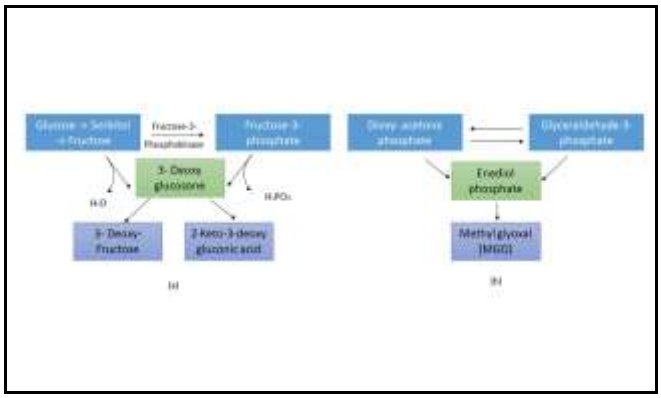

Figure-1: The Generation of Reactive Carbonyl Species via a. Polyol Pathway b. Glycolytic Pathway[8]

The question is why clinically evident nephropathy occurs in 20-50% of diabetic Patients within 5-10 years of the start of the disease.[9] Genetic variations within the CBR1 gene may modulate the enzymatic activity of carbonyl reductase-1, possibly influencing susceptibility to DN. These genetic variations can be exploited for early diagnosis of DN or risk identification. This study will also help generate a local database of the Pakistani population in NCBI.

METHODOLOGY

This case-Control study was conducted at the Center for Research in Experimental and Applied Medicine (CREAM), within the Department of Biochemistry & Molecular Biology, Army Medical College, Rawalpindi, Pakistan. The research was conducted in partnership with the Department of Nephrology at Pak Emirates Military Hospital (PEMH) and the Armed Forces Institute of Transfusion (AFIT), Rawalpindi. The study spanned from November 2020 to December 2024. The study received approval from the institutional ethics review committee (ERC) under approval number ERC/ID/96, issued on November 12, 2020. The study recruited a total of 213,[10] participants using a non-probability convenience sampling method. The sample size was calculated using the WHO formula, assuming a prevalence of 13.6%,[11] with a 95% confidence interval and a 5% margin of error. The Controls were 100, while 113 were Patients diagnosed with diabetes and experiencing advanced kidney disease within a decade of their diabetes diagnosis. All participants provided informed written consent before sample collection and enrollment in the study.

Inclusion Criteria: Patients between 18 and 80 years12 of age with a confirmed diagnosis of type 1 or type 2 diabetes were enrolled in the disease Group, provided their glycosylated hemoglobin (HbA1c) level was greater than 6.5% (48 mmol/mol). In addition, eligibility required an estimated glomerular filtration rate (eGFR) of less than 45 mL/min/1.73 m² or current treatment with dialysis.[12] The Control Group comprised healthy individuals aged 18 to 80 years with no history of diabetes, renal disease, diabetic complications, or any condition known to affect glucose metabolism. Their HbA1c levels were required to be 6.5% or lower, with normal urea and creatinine levels.

Exclusion Criteria: Participants who were unconscious, had comorbid conditions like hypertension, suffered from recent illnesses, were diabetics with other complications, or were on medications that could affect study outcomes were excluded.

For biochemical and genetic analysis, a total of 10mL of blood was collected from each participant. Venous blood was collected in BD Vacutainers, with one tube allocated for serum separation and the other collected in EDTA for genetic analysis. Serum samples were processed for biochemical investigations. Genomic DNA was isolated from EDTA-anticoagulated blood using the phenol–chloroform method; in parallel, DNA extraction was also carried out using a commercially available extraction kit (Favorgen, Taiwan).[13] The phenol-chloroform extraction,[14] involved a two-day protocol with initial lysis using prepared solutions, centrifugation, and overnight incubation with proteinase K, followed by DNA precipitation using ethanol and sodium acetate. DNA integrity was verified through 1% agarose gel electrophoresis in 1X TBE buffer, visualized using the BIO-RAD Gel Doc system. DNA quantification was performed using the Thermo Scientific Multiskan Sky Microplate Spectrophotometer with SkanIt Software 5.0. Primers for exon amplification were designed using Primer-BLAST and Primer 3 Plus, sourced from Macrogen (Seoul, South Korea). Extracted DNA was stored at -20°C for further use. The extracted DNA was subjected to polymerase chain reaction (PCR) analysis using tetra-primer amplification refractory

mutation system (ARMS-PCR) to identify the single-nucleotide polymorphism (SNP) rs533864137 in the CBR1 gene. Amplification products were analyzed through electrophoresis using a 2% agarose gel stained with ethidium bromide. A 100 base pair (bp) DNA ladder was used for band size comparison. The results and analysis of biochemical parameters are mentioned in our previously published article,[15] related to this research.

Statistical analyses were conducted using the Statistical Package for the Social Sciences software (version 22). Genotypic and allelic frequencies were assessed using SNPStat software, while the Hardy-Weinberg equilibrium was also calculated. The genotypic association with disease was assessed using the chi-square test (χ^2). Mean±SD for biochemical parameters were calculated using an independent samples t-test to ascertain significant difference in Control and disease Group biochemical parameters. The *p*-value of ≤ 0.05 was considered as significant.

RESULTS

Out of a total number of 100 in the Control Group, 97(97%) individuals had the GG genotype, and 3(3%) had the GA genotype. Among 113 Patients in the DN Group 101 were valid results, 100(99.1%) exhibited the GG genotype, while 1(0.9%) was homozygous for the AA genotype. No significant differences in genotype distribution were observed between Groups (p=0.31). Statistical analysis confirmed that no homozygous or heterozygous variant in SNP rs533864137 was significantly associated with early and severe DN as shown in Table-II.

The G allele was the predominant allele in both Groups, observed in 197 alleles (98.5%) in the Control Group and 200 alleles (99.0%) in the DN Group. In contrast, the A allele was rare, occurring in only 3 alleles (1.5%) in the Control Group and 2 alleles (1.0%) in the DN Group. There was no significant difference in allele frequencies between the two Groups (χ^2=1.04,

Table-I: Tetra Primer ARMS PCR Primers

Target SNP/ Product Size	Primers		Tm oC	Optimized at Tm oC
SNP rs533864137 A allele: 159 bp G allele: 143 bp Outer primers: 254	Forward inner	5′ACAAGTTTGTGGAGGATACAAAGAATGA3′	64	64.4
	Reverse inner	5′GCCCTCCTTCTGGTGCAATC3′	64	
	Forward outer	5′TCATGAGCGTCAGAGCCCTTAA3′	64	
	Reverse outer	5′GGCATTCAGGAGGATCTTGTCC 3′	64	

SNP: single nucleotide polymorphism, Tm: annealing temperature, oC: centigrade, bp: base pairs

Table -II: Association of SNP rs533864137 Genotypes with Diabetic Nephropathy (n=201)

Genotype (SNP rs533864137)	Frequency & Percentage of Genotype n (%)		χ^2	*p*-value
	Controls	DN Patients		
Wild Type Homozygous (GG)	97 (97.0)	100 (99.1)	1.04	0.31
Mutant Type Heterozygous (GA)	3 (3.0)	0 (0.0)		
Mutant Type Homozygous (AA)	0 (0.0)	1 (0.9)		
Total	100 (100%)	101* (100)		

**The result of 12 cases could not be determined on tetra Primer ARMS PCR*

Table-III: Allelic Association of SNP rs533864137 with Diabetic Nephropathy (n=201)

Allele	Control Group n (%)	DN Group n (%)	χ^2	*p*-value
G	197 (98.5)	200 (99.0)	1.04	>0.05
A	3 (1.5)	2 (1.0)		
Total	200 (100)	202 (100)		

Table-IV: Biochemical Parameters of the Control and DN Group (n=201)

Biochemical Parameter	Control Group (n=100)	Patient Group (n=113)	*p*- value (<0.05)
	Mean±SD	Mean±SD	
HbA1c (%)	5.35 ± 0.56	8.04 ± 1.17	< 0.01
Urea (mmol/l)	4.28 ± 1.00	23.85 ± 15.08	< 0.01
Creatinine (mmol/l)	88.23 ± 17.32	487.63 ± 252.54	< 0.01
eGFR (ml/min)	102.16 ± 23.60	14.30± 18.50	< 0.01
Albumin (g/dl)	48.24 ± 5.27	38.84 ± 7.57	0.99

**HbA1c: Glycosylated hemoglobin, DN: diabetic nephropathy, SD: standard deviation, eGFR: estimated glomerular filtration rate*

p >0.05) Shown in Table-III.

Glycemic Control, assessed by HbA1c, was significantly higher in the Patient Group (8.04±1.17%) compared with the Control Group (5.35±0.56%), indicating poor long-term blood glucose Control among Patients (p<0.01).Renal function markers were also significantly altered in the Patient Group. Serum urea levels were markedly elevated in Patients (23.85±15.08 mmol/L) compared to Controls (4.28±1.00 mmol/L), with a statistically significant difference (p<0.01). Similarly, serum creatinine was substantially higher in the Patient Group (487.63±252.54 mmol/L) than in the Control Group (88.23±17.32 mmol/L), reflecting impaired kidney function (p<0.01).However, estimated glomerular filtration rate (eGFR), an indicator of renal filtration capacity, was significantly reduced in the Patient Group (14.30±18.50 ml/min) compared with the Control Group (102.16±23.60 ml/min), further confirming severe renal dysfunction among Patients (p<0.01).Moreover, Serum albumin levels were lower in the Patient Group (38.84±7.57 g/dl; n=76) compared to the Control Group (48.24±5.27 g/dl; n=95); however, this difference was not statistically significant (p=0.99) given in Table IV.

DISCUSSION

The genetic analysis conducted in this study revealed that the wild-type GG genotype was the most prevalent across both Groups. A smaller proportion of participants exhibited heterozygous (GA) and homozygous (AA) variants, which were distributed in both the Control and DN Groups. However, statistical evaluation showed no significant differences between these Groups (p-value 0.310), suggesting that these specific genetic variations may not play a direct role in DN susceptibility.

The genotypic distribution of SNP rs533864137 in CBR1 demonstrated that among the Control Group, 97% of individuals carried the GG genotype, while 3% exhibited the GA genotype. In contrast, 88.5% of DN Patients had the GG genotype, while only 0.9% had the AA genotype. This pattern indicates that the wild-type GG remains predominant, while variant alleles appear infrequently. The absence of significant genotypic variations between cases and Controls supports previous findings that suggest CBR1 plays a role in oxidative stress regulation and metabolic pathways rather than direct DN susceptibility.[16]

Although SNP rs533864137 has not previously been reported in DN, CBR1 is recognized for its role in oxidative stress reduction and detoxification of reactive carbonyl species.[17] The findings of this study suggest that while genetic variations within CBR1 may not be directly linked to DN susceptibility, further functional analysis is required to determine their role in disease modulation. We have recently published our findings that identified novel mutations in the GLO1, ACE, and CBR1 genes associated with severe diabetic nephropathy in our Patients.[18]

While no statistically significant association was identified, these results highlight the need for further investigations in larger and more diverse populations. Future research should focus on the functional consequences of these SNPs on CBR1 enzymatic activity and their potential contribution to DN pathophysiology. Additionally, exploring gene-environment interactions may provide deeper insights into the mechanisms underlying DN progression.

LIMITATION OF THE STUDY

Due to limited funding the advanced techniques of genetic analysis could not be employed.

CONCLUSION

The SNP rs533864137 does not appear to be associated with DN in this population. These findings suggest that rs533864137 may not serve as a reliable genetic marker for assessing DN risk in the studied cohort.

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Authors Contribution

Following authors have made substantial contributions to the manuscript as under:

SZHS: Conception, study design, drafting the manuscript, approval of the final version to be published.

Authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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