

Original Paper

# Significant Association of Candidate Genes (AGTR1 and TGF- $\beta$ 1) Polymorphism with Diabetic Nephropathy in Diabetes Mellitus Type 2 Patients

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## Key Words

Type 2 diabetes mellitus • Diabetic nephropathy • AGTR1 • TGF- $\beta$ 1 • Gene polymorphism

## Abstract

**Background/Aims:** Diabetic nephropathy (DN) is one of the complications of diabetes mellitus (DM). This study aimed to investigate the association between genetic polymorphisms, specifically AGTR1 (rs5186) and TGF- $\beta$ 1 (rs1800470), and the risk of developing Diabetic nephropathy (DN) in type 2 diabetes mellitus patients, compared to those without DN and healthy controls. **Methods:** A case-control study was conducted on 165 diabetic patients (59 with diabetic nephropathy (DN) and 54 without DN (DM)), and 52 healthy controls (HC). The genotyping was done using amplification refractory mutation system method (ARMS-PCR). Age, gender, and duration of diabetes were matched across groups. Clinical parameters including FBS, RBS, HbA1C, creatinine, urea, SBP, DBP, total cholesterol, triglycerides, LDL, and BMI were assessed. **Results:** Diabetic patients with nephropathy exhibited significantly higher levels of clinical parameters compared to those without nephropathy and healthy controls. The risk allele of AGTR1, C ( $p < 0.0001$ ), and risk allele containing genotypes AC ( $p < 0.0001$ ) and CC ( $p = 0.0010$ ) were significantly higher in DN patients compared to DM and HC groups. Similarly, the TGF- $\beta$ 1 risk allele C ( $p = 0.0001$ ), and corresponding genotypes TC ( $p = 0.0038$ ) and CC ( $p = 0.0027$ ) were significantly associated with increased risk of diabetic nephropathy compared to DM and HC groups. **Conclusion:** The data showed significant association of AGTR1 (rs5186) and TGF- $\beta$ 1 (rs1800470) polymorphism with an increased risk of diabetic nephropathy in type 2 diabetes mellitus patients. More investigation will be required to disseminate the results, while increasing the samples size and using whole genome sequencing.

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## Introduction

Diabetes mellitus (DM) is a complex disorder, which is very common and rapidly growing all over the world [1]. In 2002, about 173 million cases of DM were estimated worldwide and this number was predicted to increase by 350 million in 2030 [2]. The disease is influenced by both environmental and genetic factors [3]. Diabetic Nephropathy (DN) is one of the microvascular complications of DM. It is estimated that approximately 30–40% of all patients with diabetes develop DN and is the leading cause of end stage renal disease (ESRD) [4]. The ESRD is considered the leading cause of kidney failure and death in patients with DM and that is why it is important to prevent diabetes development into DN [5]. Hyperglycemia, hyperlipidemia, hypertension, advanced glycation products accumulation, duration of diabetes, familial clustering and genetic determinants are some of the risk factors which can make a diabetic patient susceptible to DN [2].

Transforming growth factor beta (*TGF-β*) belongs to a group of multifunctional growth factors which control various biological processes such as apoptosis, senescence, healing of wounds, tumor metastasis and suppression, cell division, differentiation and immunity [6]. In humans, there are three isoforms of *TGF-β* namely *TGF-β1*, *TGF-β2* and *TGF-β3*. Among these the *TGF-β1* is the most abundant and is highly conserved in primary sequence through evolution [7]. Since the *TGF-β1* controls the production and degradation of the renal extracellular matrix (ECM), as well as the expression of cell adhesion molecule receptors, it is said that it might be directly involved in developing a kidney disease [8]. The high levels of glucose in blood activates *TGF-β1* transcription in mesangial cells. Such high glucose levels and the recombinant *TGF-β* increase the synthesis of ECM in the renal cells and also participate in cell hypertrophy [9]. The human *TGF-β1* gene is located on chromosome 19q13.1–13.3 [10] and more than 10 polymorphic loci are currently known, which are distributed in exons and introns regions as well as in the 5'- flanking region [3]. The polymorphisms at codons 10 and 25 are reported to be associated with increased or decreased levels of *TGF-β1* production *in vitro* [11]. This increase or decrease in the production of *TGF-β1* has been linked to various diseases such as atherosclerosis and fibrotic diseases of the liver, kidney and lungs [12]. Various SNPs are present in certain loci of the *TGF-β1* gene which affect its regulation and expression levels. Among such SNPs our focus in this study was on the *TGF-β1* gene polymorphism T > C (rs1800470).

Another candidate gene, Angiotensin II Receptor Type 1 (*AGTR1*) is found to be a highly polymorphic but the rs5186 (A1166C) polymorphism is greatly evaluated [13]. It has been investigated in different populations for its association with the risk and susceptibility to develop DN. Angiotensin II is one of the main enzymes of the renin-angiotensin aldosterone system (RAAS) which plays an important role in maintaining the blood pressure [14]. Hyperglycemia, which could activate RAAS, increases tissue angiotensin II that induces glomerular hyperfiltration, oxidative stress, thrombosis, endothelial damage, inflammation and vascular remodeling [15]. Studies on Animal models have also revealed that RAAS may participate in transition of epithelial cells to mesenchymal cells and thereafter renal fibrosis under hyperglycemic environment. Such studies discovered that the angiotensin II and its receptor may be a potential predictor for developing DN in patients with diabetes [16]. It has been suggested that the mutation A1166C (rs5186) of *AGTR1* gene is one of the potential candidate genes for DN. This A1166C variation is located in the 3' end of the non-coding regions and it may affect the stability and translation of the mRNA [17].

However, GWAS have identified the selected SNPs in various ethnicities with inconsistent results. Understanding the clinical implications of these genetic variants is essential for improving risk prediction, patient stratification, and therapeutic targeting in individuals with type 2 diabetes mellitus. Therefore, our study aims to investigate the presence and association of *TGF-β1* and *AGTR1* gene polymorphisms with the risk of developing DN of diabetic type 2 patients of Khyber Pakhtunkhwa, Pakistan.

## Materials and Methods

### *Study subjects, inclusion, exclusion criteria and Blood sampling*

This case control study was performed between February and December 2020 in the Institute of Biotechnology and Genetic Engineering (Health Division), The University of Agriculture Peshawar. A total of 165 participants were enrolled in the study including 59 patients with diabetic nephropathy (DN), 54 patients without nephropathy (DM) and a control group consisting of 52 healthy controls (HC). A written informed consent was obtained from each participant after describing the aim of the study. T2DM was diagnosed on the basis of WHO criteria of fasting blood glucose level of  $\geq 7.0$  mmol/L (126 mg/dL). Subjects with  $> 10$  years of diabetes duration and having urinary albumin levels  $< 30$   $\mu$ g/mg creatinine measured on two consecutive collections with no issues in the kidneys were placed in the DM group. The status of the DN was determined on the basis of clinical features, laboratory reports and questionnaires. Subjects with  $> 10$  years of diabetes duration with urinary albumin levels of  $\geq 300$   $\mu$ g/mg of creatinine in at least two of three fasting urine collections over a period of 3 months were placed in DN group. Subjects of the control group consisted of healthy participants without any history of diabetes and any kind of kidney disease. Exclusion criteria in this study were the subjects who did not belong to Khyber Pakhtunkhwa population, patients with Type 1 diabetes, duration of diabetes less than 10 years, urinary tract infection, pregnancy, smoking, hematuria, single kidney, kidney stones or any other causes of nephropathy except diabetes type 2. About 4 mL blood samples were taken in EDTA tubes for molecular study keeping in notice the standard biosafety protocol.

### *DNA isolation and PCR confirmation*

All the blood samples were processed for genomic DNA extraction using the non-enzymatic/salting out method adopted in our lab [18, 19]. For SNPs genotyping, amplification refractory mutation system (ARMS-PCR) was carried out. NCBI Primer-BLAST software was used for designing primers (Table 1). The PCR mixture of 10  $\mu$ L was prepared consisting of 5  $\mu$ L Dream Taq Green master mix (Thermo Fischer), 3  $\mu$ L of ddH<sub>2</sub>O, 1  $\mu$ L of template DNA, and 0.5  $\mu$ L of each forward and reverse primer. The PCR amplification conditions for the *AGTR1* variant were; initial denaturation at 95°C for 5 min, proceeding with 32 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, extension at 72°C for 30 s, and final extension at 72°C for 5 min. The PCR amplification conditions for the *TGF- $\beta$ 1* codon 1 (T869C) were; 95°C for 1 min followed by 30 cycles of 95°C for 15s, 65°C for 30s, and 72°C for 40s, and final extension at 72°C for 5 min. The amplified PCR products were analyzed on 2% agarose gel.

**Table 1.** Primers used for AGTR1 (A1166C) and TGF- $\beta$ 1 (T869C)

GENE		PRIMERS
AGTR1 (A1166C)	Forward Outer (Fo)	5' - GATATTGTGGACACGGCC - 3'
	Reverse Outer (Ro)	5' - GAAACTTTACTGCCCTTTG - 3'
	Forward Inner (Fi)	5' - GTCCATAAAGTAATTTTGTGAAAGC - 3'
	Reverse Inner (Ri)	5' - GGAATGTTCTCTTGCTCCTT - 3'
TGF- $\beta$ 1 (T869C)	C allele primer	5' - GCAGCGGTAGCAGCAGCG - 3'
	T allele primer	5' - AGCAGCGGTAGCAGCAGCA - 3'
	Generic primer	5' - TCCGTGGGATACTGAGACAC - 3'

### Statistical analysis

Statistical analysis was performed using SPSS software version 2.0. Descriptive statistical method was used for demographic data analysis with  $P < 0.05$  was considered significant. The data is presented as Mean  $\pm$  SD. For categorical data, the Pearson Chi Square test was used. Deviations from Hardy–Weinberg equilibrium was tested. Comparison among different study groups were performed using one-way ANOVA. Genotypic and allelic frequencies were calculated. The comparison of genotypes was performed by obtaining Odd ratios and 95% Confidence interval values using the online software called Medcalc Odd ratio calculator. The  $P > 0.05$  was considered non-significant.

## Results

### Demographic and clinical characteristics of DN, DM and HC group

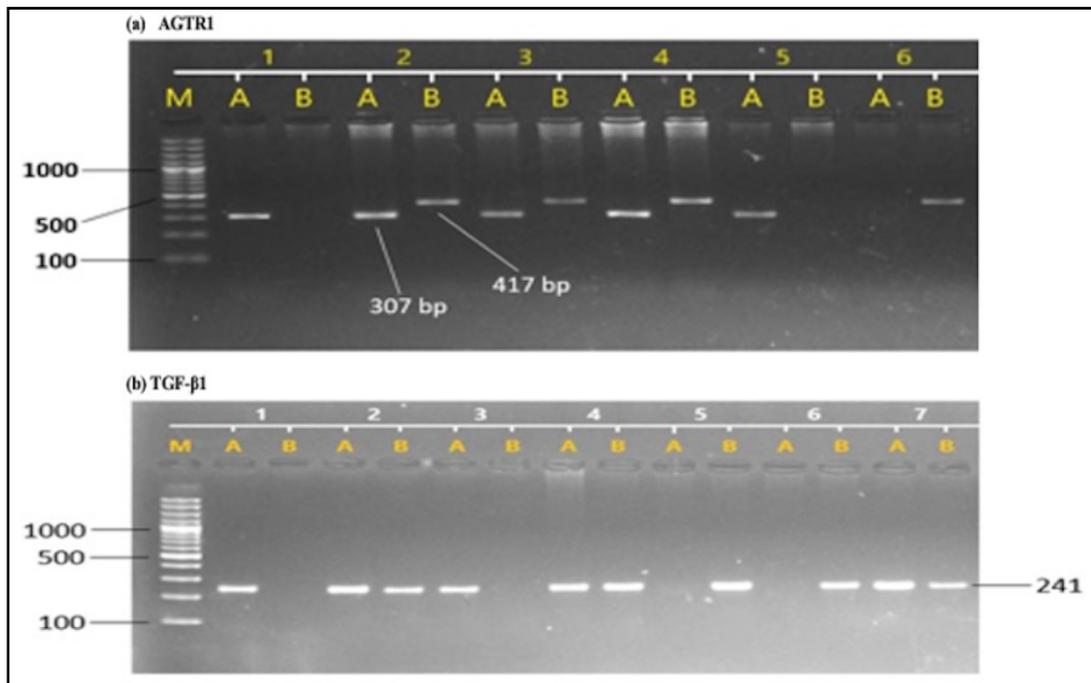
A total of 165 subjects containing 59 DN, 54 DM and 52 HC were recruited in this study. Demographic and clinical characteristics of the study subjects is summarized in Table 2. Comparison of DN group with DM and HC showed a significantly higher levels of FBS ( $P < 0.001$ ), RBS ( $P < 0.001$ ), HbA1c ( $P < 0.001$ ), Creatinine ( $P < 0.001$ ), Urea ( $P = < 0.001$ ), Systolic blood pressure ( $P < 0.001$ ), Diastolic blood pressure ( $P < 0.001$ ), Total cholesterol ( $P < 0.001$ ), Triglycerides ( $P < 0.001$ ), LDL ( $P = 0.007$ ), HDL ( $P = 0.054$ ), Hypertension ( $P = 0.023$ ), and BMI ( $P = 0.011$ ). Furthermore, the eGFR was extremely lower in the DN patients than DM and HC groups. The age, gender and duration of the diabetes showed no significant difference among the study groups (Table 2).

**Table 2.** - Demographics and biochemical characteristics of DN, DM and HC group. Data presented in Mean  $\pm$  Standard deviation. P value calculated through chi square test. DN - diabetic nephropathy, DM - Diabetes mellitus, HC - Healthy control, eGFR - estimated Glomerular Filtration Rate, LDL - low density lipids, HDL - high density lipids, BMI - Body mass index

Characteristics	DN (n=59)	DM (n=54)	HC (n=52)	P value
	Mean $\pm$ SD	Mean $\pm$ SD	Mean $\pm$ SD	
Age (years)	57.3 $\pm$ 5.60	56.8 $\pm$ 4.13	56.7 $\pm$ 2.84	0.689
Gender (F/M)	88/89	81/81	78/78	0.959
Duration of DM (years)	11.47 $\pm$ 1.017	11.25 $\pm$ 1.401	-	0.086
Fasting Blood Sugar (mg/dL)	180.0 $\pm$ 40.9	178.3 $\pm$ 35.01	87.6 $\pm$ 8.30	<0.001
Random Blood Sugar (mg/dL)	219.07 $\pm$ 35.3	215.7 $\pm$ 8.62	121.7 $\pm$ 9.02	<0.001
HbA1C (%)	8.17 $\pm$ 1.01	8.10 $\pm$ 0.78	3.76 $\pm$ 0.63	<0.001
Creatinine (mg/dL)	6.58 $\pm$ 2.31	1.06 $\pm$ 0.57	0.88 $\pm$ 0.20	<0.001
Urea (mg/dL)	153.7 $\pm$ 67.2	29.5 $\pm$ 13.2	20.10 $\pm$ 5.29	<0.001
eGFR (mL/min/1.73m <sup>2</sup> )	9.24 $\pm$ 3.78	81.09 $\pm$ 28.4	93.1 $\pm$ 17.9	<0.001
Systolic Blood Pressure (mmHg)	141.4 $\pm$ 14.47	130.4 $\pm$ 25.1	117.2 $\pm$ 8.98	<0.001
Diastolic Blood Pressure (mmHg)	90.9 $\pm$ 9.12	76.39 $\pm$ 11.46	80.8 $\pm$ 6.47	<0.001
Total Cholesterol (mg/dL)	183.8 $\pm$ 38.96	175.63 $\pm$ 35.86	144.87 $\pm$ 19.11	<0.001
Total Triglycerides (mg/dL)	148.9 $\pm$ 52.70	150.22 $\pm$ 45.49	101.25 $\pm$ 14.16	<0.001
LDL (mg/dL)	90.53 $\pm$ 28.02	84.30 $\pm$ 25.87	76.27 $\pm$ 13.02	0.007
HDL (mg/dL)	76.02	78.67 $\pm$ 22.72	85.88 $\pm$ 15.06	0.054
Hypertension	63 (35.5)	27 (16.7)	-	<0.001
BMI (Kg/m <sup>2</sup> )	24.35 $\pm$ 2.83	25.35 $\pm$ 1.57	25.45 $\pm$ 0.67	0.011

*Allelic and genotypic frequencies of TGF-β1 (rs1800470) and AGTR1 (rs5186) in the study groups*

The allelic and genotypic frequencies of *AGTR1* (rs5186) and *TGF-β1* (rs1800470) was confirmed using ARMS-PCR, run on 2% agarose gel compared with 1KB DNA ladder (Fig. 1). Our results indicated that the *AGTR1* (rs5186) risk allele C and risk allele containing genotypes AC and CC showed significant association with the increase risk of diabetic nephropathy compared with DM and HC groups (DN vs. HC, C-  $p < 0.0001$  AC- $p < 0.0001$ , CC-  $p = 0.0010$ ; DN vs. DM, C-  $p = 0.0001$ , AC-  $p = 0.0003$ , CC-  $p = 0.0022$ ; DM vs. HC, C-  $p = 0.2055$ , AC-  $p = 0.1182$ , CC-  $p = 0.8673$ ) (Table 3). Similarly, the *TGF-β1* (rs1800470) risk allele C and risk allele containing genotypes TC and CC were significantly associated with increased risk of diabetic nephropathy (DN vs. HC, C-  $p < 0.0001$ , TC- $p < 0.0038$ , CC-  $p = 0.0027$ ; DN vs. DM, C-  $p = 0.0123$ , TC-  $p = 0.0328$ , CC-  $p = 0.0282$ ; DM vs. HC, C-  $p = 0.1415$ , TC-  $p = 0.2983$ , CC-  $p = 0.2903$ ) (Table 3).



**Fig. 1.** Sample gel pictures of T869C of TGF-β1 and A1166C of AGTR1 gene polymorphism. (a), AGTR1 polymorphism, M represents DNA ladder, A represent wild allele and B represents mutant allele, sample 1 represents AA genotype, sample 2 represents AC genotype and samples 6 represents CC genotype. (b), TGF-β1 polymorphism, M represents DNA ladder, A represent wild allele and B represents mutant allele, sample 1 represents TT genotype, samples 2 represents TC genotype and samples 5 represents CC genotype.

**Table 3.** Association of AGTR1 and TGF-β1 Gene polymorphism with DN, DM and HC group. HWE - Hardy Weinberg equilibrium, OR - Odd ratios, CI - Confidence interval, χ<sup>2</sup> - Chi square value

Gene/SNP	Genotype/Allele	DN (n = 59)	M (n = 52)	HC (n = 58)	χ <sup>2</sup>	DN vs. HC	P value	DM vs. HC	P value	DN vs. DM	P value	
AGTR1 rs5186 (A1166C)	AA	12 (20.3)	32 (59.2)	38 (73.1)	<0.001	OR (95%CI)		OR (95%CI)		OR (95%CI)		
	AC	37 (62.7)	20 (37.0)	12 (23.06)		Reference		Reference		Reference		
	CC	10 (16.9)	02 (3.70)	02 (3.84)	9.76(3.89-24.48)		1.97 (0.84-4.66)		4.93(2.09-11.63)			
	HWE	0.1453	0.844	0.739	15.83(3.03-82.5)		0.0010		1.18 (0.15-8.91)		0.8673	
	A	61 (51.6)	84 (77.7)	88 (84.62)	<0.001	Reference		Reference		Reference		
C	57 (48.3)	24 (22.3)	16 (15.38)	5.13 (2.70-9.78)		<0.0001		1.57 (0.78-3.16)		0.2055		
TGF-β1 rs1800470 (T869C)	TT	14 (23.72)	25 (46.3)	30 (57.70)	0.004	Reference		Reference		Reference		
	TC	30 (50.85)	21 (38.9)	18 (34.6)		3.57 (1.50 - 8.46)		0.0038		1.54 (0.67 to 3.52)		0.2983
	CC	15 (25.43)	08 (14.8)	04 (7.70)	7.0 (1.96 - 24.98)		0.0027		3.48(0.34 to 35.22)		0.2903	
	HWE	0.991	0.603	0.857	Reference		Reference		Reference		Reference	
	T	58 (49.15)	71 (65.75)	78 (75)	<0.001	Reference		Reference		Reference		Reference
C	60 (50.85)	37 (34.25)	26 (25)	3.10 (1.75 - 5.49)		0.0001		1.56 ( 0.86 to 2.83)		0.1415		
										1.98(1.16 - 3.39)	0.0123	

## Discussion

*TGF-β* is said to be an important regulator in the production and degradation of the renal extracellular matrix [9]. When it is overexpressed, it could cause tissue fibrosis which eventually leads to failure of the organ [20]. Extreme accumulation of extra cellular matrix and the renal hypertrophy could occur because of higher concentration of *TGF-β1*, such changes progress to DN [21]. Diabetic patients were found to have an elevated renal production of *TGF-β* protein [9]. Some other factors that are known to be involved in the initiation and progression to DN are excessively increased blood sugar levels, renin angiotensin system activation, increased intraglomerular pressure and hypertension, which are said to induce the production of *TGF-β* in the kidneys [22].

The actual mechanism to know how exactly the gene polymorphism in *TGF-β1* affect the susceptibility to DN is not very clear yet. It has been known that the gene *TGF-β1* is located on the chromosome 19 (q13.1-13.3) and has a total of 7 exons and 6 introns [23]. Various polymorphisms have been identified in the *TGF-β1* gene [24]. One of them is the rs1800470, which occurs in exon number 1, at position 869. Here in this study, we have investigated *TGFβ-1* gene as a candidate gene for susceptibility to DN in patients with type 2 diabetes of Khyber Pakhtunkhwa, Pakistan. Our analysis indicated the association between allelic and genotypic frequencies of the variant rs1800470 and susceptibility to DN. The CC genotypes and C allele were more frequent in DN group as compared to DM and HC. The increase in TC genotype in patients with DN showed that it could be a risk factor of developing DN in individuals with prolonged history of diabetes. Our results are similarly with a case control study, which was carried out on Chinese patients who had a history of having diabetes mellitus type 2 for over 10 years. Their result suggested that the *TGF-β1* gene polymorphism is associated with susceptibility to diabetic nephropathy [22]. Another similar study was carried out in patients with type 1 diabetes to see if it contributes to genetic predisposition to diabetic nephropathy [7]. A study on patients of Egyptian origin and their data showed that C allele and C allele containing genotypes might be susceptible and the T allele and TT genotypes may be protective factors [3]. These results were in accordance with our findings. However, some studies reported results which were in contrast with our study. Similar study conducted on type 2 diabetes patients, in order to see the association of the *TGF-β1* gene polymorphism with diabetic nephropathy. Their results suggested that *TGF-β1* gene polymorphism is not associated with the progression of diabetic nephropathy [25]. A study on German populations concluded that the T allele rather than C allele was associated

with ESRD susceptibility in patients with type 2 diabetes [26]. However, they recommend more investigation will be required to figure out the exact association while using next generation sequencing with increase samples size.

Here, we have also investigated the association of *AGTR1* (rs5186). The *AGTR1* gene is said to be located on the 3q21-25, having a length of less than 55 kb. This angiotensin II receptor actually regulates the level of an enzyme angiotensin II, which has great important in RAAS. RAAS is a system that regulates vasoconstriction, sodium reabsorption and the inflammatory cascade, which is believed to put a positive effect on the development of DN. Several studies have been done on *AGTR1* gene polymorphisms, especially on rs5186 [17]. Our results indicated the association of *AGTR1* gene polymorphism with susceptibility to DN. We noted significantly higher level of risk allele C in the DN group as compared to those in DM and HC group. The AC genotype was also higher in DN group than DM and HC group. Similarly, a study from North Indian origin stated that C allele and CC genotype of *AGTR1* gene was significantly higher in patients with DN and the patients with AC genotype and CC genotype were found to be associated with increased risk for developing DN as compared to patients with AA genotype [27]. Another study conducted and observed the association of the *AGTR1* genotype with the development of renal disease and progression to ESRD [24]. A significant difference in the frequency of the C allele and CC genotypes between patients and control group was noted. However, a [28] case control study conducted by [29] found no significant difference in the frequency of *AGTR1* genotypes between the patients and the control group. Their study revealed a higher but not a significantly different frequency of *AGTR1* AC+CC genotype in patients as compared to controls. A Meta-Analysis study of was conducted on eight articles, four were conducted in patients of Caucasian origin, and four were in Asians. They did not find an association of *AGTR1* gene polymorphism and ESRD risk in Caucasians and Asians [30]. Therefore, to disseminate the investigation more similar research will be required to figure out the exact association of the *AGTR1* gene polymorphism with DN in our population.

## Conclusion

In conclusion, our research suggested an association of allelic and genotypic frequencies of the variant rs5186 of *AGTR1* and rs1800470 of *TGF-β1* as an increased risk of diabetic nephropathy. However, one of the short comings of this study was the small sample size. These results should be confirmed in larger sample size as well as in different ethnic groups while using modern sequencing technologies.

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### *Authors' Contributions*

MI did the experimental work and wrote the first draft under the supervision of NUK. MI, NA, NUK, MHA, IA and BDA help in data analysis and critically review the final manuscript.

### *Ethical approval and consent to participate*

Ethical approval was taken from the Institute of Biotechnology and Genetic Engineering (IBGE), The University of Agriculture Peshawar, Pakistan under (IBGE, UAP/2021/002). A written informed consent was taken from all the participants after explaining the aim of the study.

### *Authors Declaration*

All the authors approved the final manuscript and agreed to publish it

### *Consent to publish*

All the authors have read and approved the article for publication

### *Availability of data and materials*

All the necessary data are included in manuscript, related data will be provided on request from corresponding author

### **Disclosure Statement**

The authors declared no competing interests

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