

## Observational Study

# Comprehensive genetic screening reveals wide spectrum of genetic variants in monogenic forms of diabetes among Pakistani population

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## Abstract

### BACKGROUND

Monogenic forms of diabetes (MFD) are single gene disorders. Their diagnosis is challenging, and symptoms overlap with type 1 and type 2 diabetes.

### AIM

To identify the genetic variants responsible for MFD in the Pakistani population

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and their frequencies.

## METHODS

A total of 184 patients suspected of having MFD were enrolled. The inclusion criterion was diabetes with onset below 25 years of age. Brief demographic and clinical information were taken from the participants. The maturity-onset diabetes of the young (MODY) probability score was calculated, and glutamate decarboxylase ELISA was performed. Antibody negative patients and features resembling MODY were selected ( $n = 28$ ) for exome sequencing to identify the pathogenic variants.

## RESULTS

A total of eight missense novel or very low-frequency variants were identified in 7 patients. Three variants were found in genes for MODY, *i.e.* *HNF1A* (c.169C>A, p.Leu57Met), *KLF11* (c.401G>C, p.Gly134Ala), and *HNF1B* (c.1058C>T, p.Ser353Leu). Five variants were found in genes other than the 14 known MODY genes, *i.e.* *RFX6* (c.919G>A, p.Glu307Lys), *WFS1* (c.478G>A, p.Glu160Lys) and *WFS1* (c.517G>A, p.Glu173Lys), *RFX6* (c.1212T>A, p.His404Gln) and *ZBTB20* (c.1049G>A, p.Arg350His).

## CONCLUSION

The study showed wide spectrum of genetic variants potentially causing MFD in the Pakistani population. The MODY genes prevalent in European population (*GCK*, *HNF1A*, and *HNF4a*) were not found to be common in our population. Identification of novel variants will further help to understand the role of different genes causing the pathogenicity in MODY patient and their proper management and diagnosis.

**Key Words:** MODY; Diabetes; Genetics; Monogenic diabetes; Monogenic forms of diabetes

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**Core Tip:** There was a lack of data on monogenic forms of diabetes (MFD) from Pakistan, therefore this study was designed to determine the genetic variants responsible for MFD in the country. The study identified wide spectrum of genetic variants potentially causing MFD. The identification of novel variants paved the way for better understanding of genetic landscape and risk factors of MFD in the country.

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## INTRODUCTION

Monogenic forms of diabetes (MFD) result from changes in single gene. Maturity-onset diabetes of the young (MODY) is a monogenic form of diabetes. It is inherited in an autosomal dominant pattern[1] and is often misdiagnosed as type 1 or type 2 diabetes[2]. It is estimated that MODY accounts for 1%-2% of all the diabetic cases[3]. There are fourteen sub-types of MODY listed in OMIM(On-Line Mendelian Inheritance in Man) (#606391). Most common of them are *GCK*, *HNF1A*, and *HNF4A*. The other listed genes are *PDX1*, *HNF1B*, *NEUROD1*, *KLF11*, *CEL*, *PAX4*, *INS*, *BLK*, *ABCC8*, *KCNJ11*, and *APPL1*[1]. Mutations in a number of additional genes are also known to cause diabetes.

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The subtypes of MODY differ with respect to hyperglycemia, age of disease onset, treatment pattern, and complications reported[4]. Therefore timely diagnosis is of vital importance in MFD. Previously, most common genes, *i.e.* GCK, HNF1A, and HNF4A were generally sequenced for suspected cases, but now with the advent of latest technologies, targeted panel sequencing or exome sequencing are standard[5,6].

Pakistan has a huge burden of diabetes. The recent surveys showed that one out of every four people in the general population is suffering from diabetes in the country[7, 8]. Being a resource poor country, advanced diagnostic facilities are not available to the public. The literature from Pakistan is scarce on MFD[9,10]. Therefore, this study was designed to enroll suspected MFD patients and assess the causal variants involved. To our knowledge, this was the first comprehensive study to be conducted in Pakistan on MFD.

## MATERIALS AND METHODS

A total of 184 patients with diabetes onset before 25 years of age were considered for participation in the study. The participants were chosen from the sources indicated below:

### **National Diabetes Survey (n = 80)**

The record of patients who had an onset of disease before 25 years of age was obtained from National Diabetes Survey data. This was a population-based survey carried out all over the four provinces of Pakistan. The detailed methodology is described elsewhere[7]. According to World Health Organization, the patients were diagnosed as diabetic if having fasting glucose level > 126 mg/dL (7.0 mmol/L) or HbA1c > 6.5% (48 mmol/mol). Clinical information in 34 cases suggested MFD [young onset, low body mass index (BMI), mild hyperglycemia]. The glutamate decarboxylase 65 (GAD-65) ELISA testing was negative in 5 cases, which were selected for exome sequencing.

### **Prospective enrollment from Lahore (n = 15)**

A total of 15 patients were enrolled from The Diabetes Clinic at PHRC Research Centre, Fatima Jinnah Medical University, Lahore. The inclusion criteria were onset of diabetes before 25 years of age with preferential first-degree family history of diabetes. The demographic information along with history of disease (onset, complications, treatment details and family history) was collected on proforma. On the basis of BMI and preserved fasting C-peptide, GAD-65 ELISA was performed on three samples where serum was available. The two that were found negative for GAD-65 and, along with another three patients having clinical features like low BMI but for whom serum was not available, were finally selected for exome sequencing. All the selected subjects had normal c-peptide values (range: 0.8-3.8 ng/mL)

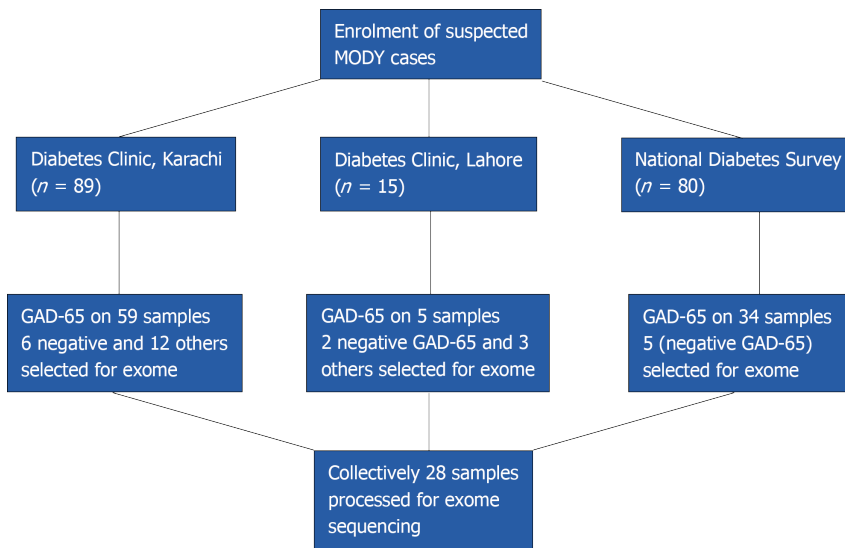
### **Prospective enrollment from Karachi (n = 89)**

The patients coming to the diabetes clinic for type-1 and type 2 diabetes treatments were enrolled if onset of the disease was below 25 years of age, with family history of diabetes as preference. Information on demography, treatment, and diagnosis was taken on pre-designed proforma. Height and weight were recorded for BMI. The GAD-65 ELISA was performed on 59 patients. Patients that were GAD-65 negative and additional patients with low BMI but no available serum for testing were selected for exome sequencing (n = 18) (Figure 1).

A total of 28 patients were selected for exome sequencing. Their median age at diagnosis was 18 years and median BMI was 22. Among them, 17 were taking treatment (14 insulin and 3 were taking OHA agent) and 5 were not taking any treatment.

The DNA was extracted using a QIAamp DNA mini kit (QIAGEN). The GAD-65 autoantibody test was done by using a KRONUS Elisa kit. C-peptide test was commercially tested in a diagnostic laboratory. Exome sequencing was performed by Genome Quebec, Canada. Exome sequencing was carried out with 50 Mb Agilent Sure select array and sequencing on Illumina Hi-seq at 50 × depth.

Written informed consent was taken from participants/parents/guardians prior to enrollment. The Ethical Clearance was taken from Institutional Bioethics Committee of Pakistan Health Research Council and Ethical Review Board of Pakistan Institute of Medical Sciences, Shaheed Zulfiqar Ali Bhutto Medical University, Islamabad.



**Figure 1** Flow chart for enrollment of patients. GAD-65: Glutamate decarboxylase.

### Data analysis

For bioinformatics analysis, the FastQ files were processed using the best practices recommendations of the genome analysis tool kit (GATK). The reads were mapped to human reference genome GRCh37 using Burrow-Wheeler alignment (BWA-MEM). The Picard tool was used to mark and remove duplicate alignments and then indel realignment and base quality score recalibration was done. The gVCFs were generated with GATK HaplotypeCaller and joint variant calling was done. The variants with low map reads ( $DP < 20$ ) and low genotype quality ( $GQ < 20$ ) were discarded. Annovar was used to annotate the variants. UTR, synonymous, and intronic (unless splicing) variants were discarded by standard procedure to focus on protein altering ones. These variants were filtered for frequency in three public databases (Exome Aggregation Consortium, 1000 genomes, and gnomAD version v2.1.1). For dominant genes, maximal allele frequency cutoff was 0.0001 in any population while it was 0.005 for recessive genes (ACMG PM2 criterion). The missense variants were selected only if predicted disease-causing by the majority of 10 algorithms used (see legend to Table 1) which satisfies PP3 by ACMG/AMP criteria). The computational evidence supports deleterious effects on the gene[11]. Analysis of the exome focused on the genes listed in the University of Chicago monogenic diabetes panel (<https://dnatesting.uchicago.edu/tests/monogenic-diabetes-panel>). Variant coordinates were searched for functional domains in uniprot.org (results shown in Table 1).

## RESULTS

A total of 80 cases selected from the National Diabetes Survey with the criteria of having diabetes and onset of disease before 25 years of age. The median age was 24 years. Most of them (62%) were females and average BMI was 28 kg/m<sup>2</sup> (Table 2). Of them, based on clinical criteria and negativity for antibody, five were selected for exome sequencing. The results revealed three novel missense variants identified in three patients. Two of the variants belong to the OMIM-listed MODY genes, *i.e.* *HNF1A* (c. 169C>A, p.Leu57Met) and *KLF11* (c. 401G>C, p.Gly134Ala) and one in *RFX6* (c. 919G>A, p.Glu307Lys), a gene recently described as mutated in dominant, MODY-like diabetes.

In the prospective enrollment from Lahore, 15 patients were enrolled. The mean age for onset of disease in patients was 23 years and mean BMI was 21.5 kg/m<sup>2</sup>. The fasting blood glucose on average was 239.26 mg/dL. The HbA1c ranged from 6% to 11% (Supplementary Table 1). The exome analysis revealed a variant in *HNF1B* (c. 1058C>T, p.Ser353Leu) for one patient.

In the prospective enrollment from Karachi, 89 diabetic patients were enrolled. The mean age of onset of disease was 16.5 years and the current age was 26.2 years. Mean BMI was 24.3 kg/m<sup>2</sup>. Most of them (95%) had family history of diabetes. Among them, 93% were taking treatment for diabetes and 68% were taking insulin. About 25% of the

**Table 1 Rare variants in cases of monogenic forms of diabetes**

Case ID	Chromosome position	Gene symbol	cDNA change	Protein change	Maxfreq.	In silico prediction	Protein region byUniport (uniprot.org)
612	12; 1416740	<i>HNF1A</i>	c. 169C>A	p.Leu57Met	0	T, NA, D, D, N, L, D, D, D, D	DNA-interacting
705	2; 10187916	<i>KLF11</i>	c. 401G>C	p.Gly134Ala	0	T, N, N, T, N, N, T, T, T, N	NA
830	6; 117237424	<i>RFX6</i>	c. 919G>A	p.Glu307Lys	0.0001	T, D, D, T, N, N, T, T, D, D	NA
P-9	17; 6070581	<i>HNF1B</i>	c. 1136C>T	p.Ser379Leu	0.00003	NA, D, D, NA, NA, L, D, D, D, D	NA
P-17	4; 6292941	<i>WFS1</i>	c. 478G>A	p.Glu160Lys	0	T, N, N, D, N, M, D, D, D, D	NA
	4; 6292980		c. 517G>A	p.Glu173Lys	0	D, D, D, D, D, M, T, T, D, D	
P-68	6; 117241502	<i>RFX6</i>	c. 1212T>A	p.His404Gln	0.0001	T, D, D, T, N, L, T, T, T, D	NA
P-87	3; 114069876	<i>ZBTB20</i>	c. 1049G>A	p.Arg350His	0	D, D, D, T, N, N, T, T, T, D	NA

Fathmm-MKL\_coding are listed in sequence. D: Deleterious; T: Tolerated for SIFT, LRT, MetaSVM, MetaLR\_pred, M-Cap prediction, FATHMM; N: Neutral for LRT and Provean. For mutation assessor, H: High; M: Medium; L: Low; N: Neutral; NA: Not available. SIFT: Sorting intolerant from tolerant; LRT: Likelihood ratio test; Mutation taster, FATHMM: Functional Analysis through Hidden Markov Models; PROVEAN: Protein Variation Effect Analyzer; Mutation assessor, MetaSVM: Support vector machine; MetaLR: Logistic regression; M-CAP: Mendelian Clinically Applicable Pathogenicity.

**Table 2 Demographic features of patients having causal variants**

ID	Age at diagnosis	Sex <sup>1</sup>	HbA1c (%)	Affectedparent <sup>2</sup>
612	25	F	9.2	M and F
705	Not listed but current age 23	M	5.4	NA
830	Not listed but current age 21	F	7.2	NA
P-9	20	M	6.20	-
P-17	15	F	NA	M
P68	13	M	6.8	M
P87	25	M	6.10	M

<sup>1</sup>M: Male; F: Female.

<sup>2</sup>M: Mother; F: Father.

patients had a MODY probability score more than 75%. The serum was available for 59 patients. The GAD-65 autoantibody test was conducted on all patients and 15 of them were negative. The patients whose serum was not available were shortlisted on the basis of low BMI, family history, and HbA1c levels.

Among them, 18 were selected for exome sequencing, which revealed potentially causal variants in three patients (Table 1). One had a variant in *RFX6* (c. 1212T>A, p.His404Gln) and a second one was a compound heterozygote (Supplementary Figure 1) for *WFS1* (c. 478G>A, p.Glu160Lys and c. 517G>A, p.Glu173Lys). Recessive *WFS1* mutations cause Wolfram syndrome, but non-syndromic diabetes alone is also seen[12]. A third patient had a variant in *ZBTB20* (c. 1049G>A, p.Arg350His).

The mutation in this gene is responsible for causing primrose syndrome[13]. The patient did not have the other manifestations of this syndrome.

All variants reported here were missense, all satisfied the PP3 and PM2 criteria but, being novel and not having previously been reported, all classified as VUS. Nevertheless, our extremely low cut-off for allele frequency of 0.0001 (more than two orders of magnitude lower than the ACMG/AMP cut-off for a VUS), minimizes the

probability of spurious variants. The MODY probability score was calculated for all the participants. More than half of the patients in National Diabetes Survey, all patients from Lahore and one fourth of patients from Karachi had probability scores more than 75%. The probability score was calculated by MODY probability calculator developed for Caucasian population.

## DISCUSSION

Exome sequencing of 28 suspected patients for MFD identified missense novel variants in 7 patients (with the caveat that *KLF11*, *BLK*, and *PAX4* are not universally accepted as genes whose mutation causes diabetes). Previous studies from Pakistan have discussed the importance of diagnosing MFD in Pakistan[9,10]. However, to the best of our knowledge, this is the first comprehensive study from the country to enroll suspected MFD patients for exome sequencing.

We enrolled diabetic patients with early onset of disease below 25 years of age with clinical features suggestive of MFD and negative (or unknown) for GAD65 auto antibodies. The probability score was calculated by using the MODY probability calculator developed for the Caucasian population[14]. However there is a need to validate this with South Asian populations[15].

In determining the type of MFD, ethnic differences play an important role. There is wide variation of prevalence of different MFD types in different areas. The *HNF1A* MODY type was reported to be more prevalent in northern Europe while *GCK* MODY types in southern Europe[16]. There were similar findings from United States regarding the three major prevalent types of MODY in their population[17]. The study on Russian children with non-type 1 diabetes showed that the most prevalent MODY type was *GCK*, with only 18% of variants in other than known MODY genes. The studies from China reported that *HNF4A* MODY types were relatively less common as compared to Europeans[18,19]. A study from Korea also has a similar finding that common MODY types prevalent in Europe were not common there, but instead they found variants in three new genes, including the *WFS1* gene[20]. The MODY landscape in India is also complex, reporting MODY types other than common genes known in European population[21,22]. A study from Oman reported that variants were not found in three common MODY genes[23]. Similar findings were also reported from Tunisia that common MODY types were not found there and concluded that other genes might be responsible for young onset diabetes in their population[24, 25]. These discrepant results may be only partially explained by different methodologies and different selection criteria for testing.

We found in our study in a total 28 screened patients that 3 of the patients tested had variants in OMIM-listed MODY genes while 4 had variants in other genes also known to be mutated in MFD. Similar findings were also reported from Norway [26], France[27] and Sweden[28]. Two novel missense variants were found in *RFX6* (c.G919A, p.E307K). In addition to Europeans[29], variants from this gene was also reported in studies from India[22] and Japan[30]. One patient was compound heterozygous at *WFS1*, the gene mutated in Wolfram syndrome and also responsible for non-syndromic diabetes. *WFS1* variants have been reported from India[22], China[12,31], Korea[20,32,33], Russia[6], and European ancestry[34]. Finally, one variant was identified in *ZBTB20*, a transcription factor that regulates the function of beta cells and glucose homeostasis[35-37]. In humans, dominant *ZBTB20* mutations cause Primrose syndrome.

The three variants in OMIM-listed MODY genes were found in *HNF1A*, *KLF11*, and *HNF1B*. The *HNF1A* (MODY 3) is most common type prevalent in some European and Asian countries[38] and variants in this gene have been reported from various countries all around the world[5,39,40]. The patients with *HNF1A* variants respond well to sulfonylurea therapy[41]. The other variant was found in the *KLF11* (MODY 7) [42]; variants from this gene have been reported from France[43] and Japan[44], although recent literature disputes this gene as true MODY gene. One variant was found in *HNF1B* (MODY 5) as reported to account for 2%-6% of all the diagnosed MODY cases[45]. This type was generally found to be associated with kidney dysfunction[46]. Variants in this gene have been reported from different countries[47-49]. Although it has been considered that *KLF11*, *BLK*, and *PAX4* are not MODY causing genes, the OMIM and recent literature reported it as involved in MODY[3,6].

Pakistan, being a developing country, is facing a huge burden of diabetes as evident from the recent survey findings that 26% of adults in the general population were suffering from diabetes[7]. There is a need to identify the genetic basis of the diabetes

in Pakistan, with large-scale efforts of screening. As Pakistan is a limited-resource society, it is important to develop sensitive and population-specific criteria. We propose our paper contributes as the first step in this direction.

A study reported that 56% of the marriages were consanguineous, and among them, over 49% were first cousin marriages[50,51], suggesting unknown recessive genes, such as seen in non-syndromic *WFS1* cases. Studies from Pakistan on MFD were very scarce. According to American Diabetes Association, the diagnosis of MFD should be considered when there is a family history of diabetes with atypical features of diabetes, such as lacking obesity[52]. There is a need to conduct large scale genetic studies on young onset diabetes to understand the genetic aspects from our country.

## CONCLUSION

A wide spectrum of genetic variants involved in MFD was identified from this study. The common genes prevalent in European countries were not found common in this study. The genes other than commonly known MODY genes were identified. There is need for large scale genetic studies on early onset of diabetes in the country.

## ARTICLE HIGHLIGHTS

### Research background

The data on monogenic forms of diabetes (MFD) was lacking from Pakistan.

### Research motivation

The identification of MFD from Pakistan will paved the way for better diagnostics and treatment for patients

### Research objectives

To identify the genetic variants for MFD.

### Research methods

Exome sequencing was used.

### Research results

The wide spectrum of genetic variants was identified.

### Research conclusions

The MODY genes prevalent in other countries, like those in Europe, were not found common in our population

### Research perspectives

More studies are required, keeping in view the consanguinity rate in Pakistan

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