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Molecular characterization and re-interpretation of *HNF1A* variants identified in Indian MODY subjects towards precision medicine

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Background: *HNF1A* is an essential component of the transcription factor network that controls pancreatic β-cell differentiation, maintenance, and glucose stimulated insulin secretion (GSIS). A continuum of protein malfunction is caused by variations in the *HNF1A* gene, from severe loss-of-function (LOF) variants that cause the highly penetrant Maturity Onset Diabetes of the Young (MODY) to milder LOF variants that are far less penetrant but impart a population-wide risk of type 2 diabetes that is up to five times higher. Before classifying and reporting the discovered variations as relevant in clinical diagnosis, a critical review is required. Functional investigations offer substantial support for classifying a variant as pathogenic, or otherwise as advised by the American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP) ACMG/AMP criteria for variant interpretation.

Objective: To determine the molecular basis for the variations in the *HNF1A* gene found in patients with monogenic diabetes in India.

Methods: We performed functional protein analyses such as transactivation, protein expression, DNA binding, nuclear localization, and glucose stimulated insulin secretion (GSIS) assay, along with structural prediction analysis for 14 *HNF1A* variants found in 20 patients with monogenic diabetes.

Results: Of the 14 variants, 4 (28.6%) were interpreted as pathogenic, 6 (42.8%) as likely pathogenic, 3 (21.4%) as variants of uncertain significance, and 1 (7.14%) as benign. Patients harboring the pathogenic/likely pathogenic variants were able to

successfully switch from insulin to sulfonylureas (SU) making these variants clinically actionable.

Conclusion: Our findings are the first to show the need of using additive scores during molecular characterization for accurate pathogenicity evaluations of *HNF1A* variants in precision medicine.

KEYWORDS

Maturity Onset Diabetes of Young (MODY) subtype-3, acmg-amp guidelines, re-interpretation, pathogenic variants, functional characterization, structural analysis, ACMG-AMP guidelines

1 Introduction

The hepatocyte nuclear factor 1A (***HNF1A***)gene (MIM # 142410) encodes a crucial member of an auto-regulatory transcription circuit in mature and developing pancreas. Heterozygous mutations in *HNF1A* result in the most common form of MODY namely subtype *HNF1A*-MODY. Autosomal dominant inheritance, early onset, and progressive β-cell deterioration resulting in severe hyperglycemia define this type of monogenic diabetes (1–3). This kind of MODY has the highest prevalence and is more common than other subtypes, and it is more common in Europe, North America, and Asia (4–7).

Individuals with *HNF1A* MODY are likely to develop extra pancreatic symptoms such as glycosuria which will appear even before the onset of diabetes due to a low renal glucose threshold (8). This is mainly because *HNF1A* is expressed in tissues such as the kidney, liver, and small intestine, in addition to β-cells. The risk of micro- and macro-vascular problems in *HNF1A*-MODY is comparable to that of T1D and T2DM (9) and hence strict glucose management is required for these individuals. Patients harboring pathogenic variants in *HNF1A* gene are sensitive to low doses of sulfonylureas (10).

The *HNF1A* protein consists of three functional domains namely a dimerization domain (1 – 33 aa), a bipartite DNA-binding domain (homeo domain 100 –184 aa; POU domain 198 –281 aa), and a transactivation domain (282 –631 aa) (11, 12). It binds to DNA as a homodimer or with the structurally related transcription factor *HNF1B* as heterodimers (13, 14). To date, about 564 MODY-causing variants have been identified in the *HNF1A* gene (15, 16). These variations include missense, nonsense, frameshift, in-frame deletions/insertions/duplications, splice site, promoter region, and whole/partial gene deletions. Analyses of these variants have demonstrated that some of them render the protein unstable and poorly expressed (17, 18). Some of the variants affect either the DNA binding or transactivation ability of *HNF1A*. However, patients with the latter type of variants do not exhibit more severe phenotypes (19–21). Finally, a subgroup of variants exert a dominant-negative effect over the normal protein.

It is important that these candidate variants are subjected to rigorous evaluation of pathogenicity to avoid false annotation of causality, which would be an impediment to the translation of genomic research findings to clinical practice and precision medicine. False assignment of pathogenicity can also have severe consequences for patients, resulting in incorrect prognostic and therapeutic advice. Therefore, a comprehensive map is needed, linking mutation status, effect on protein function, and clinical effect that is genotype-function-phenotype. The recent American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP) (ACMG-AMP) guidelines classification is based on five tier score system namely pathogenic (P), likely pathogenic (LP), variant of uncertain significance (VUS), likely benign (LB) and benign (B) (22). Our previous studies have shown that *HNF1A* -MODY is the most prevalent subtype in India (3) and we identified several variants which were of uncertain significance, Assessing the pathogenicity of these rare protein-coding genetic variants in *HNF1A* is very important in our patient cohort before assigning causality to these variants, as this may lead to change of treatment.

Functional investigation constitutes one of the strongest pieces of evidence for classifying a variant as pathogenic or benign (23). Each variant needs to be assessed by genomic, bioinformatic, structural, and functional lines of evidence for classifying them as pathogenic or benign. Hence, we hypothesized that functional evaluation would enhance the interpretation of the pathogenicity of *HNF1A* variants identified in individuals from families of Indian MODY subjects.

2 Materials and methods

2.1 Subjects

We investigated 14 *HNF1A* variants found in 20 unrelated individuals (11 females and 9 males) from 20 non-consanguineous Indian families. Patients were selected for MODY genetic screening based on the following criteria: a family history of diabetes in multiple generations; an early age at onset of diabetes (< 35 years); lack of obesity, ketosis, and beta cell autoimmunity with detectable endogenous insulin reserve as measured by C peptide which is one of the best biomarkers; and diabetes controllable without insulin for at least 2 years. The study was carried out in compliance with the

Helsinki Declaration (2000); all study participants (or their guardians) provided written, informed consent, and the study was approved by the Madras Diabetes Research Foundation's local institutional ethics committee.

2.2 Genomic analyses

Genomic DNA was isolated from whole blood using the standard protocol. Direct sequencing was carried out on an ABI 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA) using the Big Dye terminator V3.1 chemistry, and the sequences were compared with the public databases. Published primer sequences were used to amplify the DNA for *HNF1A* gene. In addition to the sequencing of patients, we also sequenced 100 normal glucose-tolerant subjects (fasting value <100 mg/dL and 2 hours value <140 mg/dL) to check for the presence or absence of variants in them.

2.3 ACMG classification

All *HNF1A* variations were assessed using the ACMG guidelines, which classify variants as pathogenic (class 5), likely pathogenic (class 4), uncertain significance (class 3), likely benign (class 2), or benign (class 1). Criteria used for the classification of variants are listed in Supplementary Table 1. Public databases such as PubMed, the Human Gene Mutation Database, ClinVar, and LOVD were used and the genome aggregation database (GnomAD) was referred to for population frequency. Bioinformatic prediction tools such as SIFT, PolyPhen2, Mutation Taster, PROVEAN, CADD Score, i mutant 2.0, and Grantham scores were used to assess the pathogenicity (Supplementary Table 2).

2.4 Functional analysis

Human *HNF1A* cDNA (NCBI Entrez Gene BC104910.1) (NM_000545.5) in pcDNA 3.1 His/C vector (Invitrogen Inc, Carlsbad, CA, USA), was used as a template for constructing individual *HNF1A* variants using the QuikChange Lightning Site-directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA), and all constructs were verified by Sanger sequencing. Transiently transfected HeLa and INS1 cells with WT, empty vector (pcDNA3.1), or variant *HNF1A* cDNA were used in functional studies, investigating *HNF1A* (i) transcriptional activity using a rat albumin (in HeLa cells) and *HNF4A P2* (in INS1 cells) promoter-linked luciferase reporter assay system; (ii) DNA binding ability was analyzed using Episeeker DNA-protein binding assay kit (Abcam, ab117139) and a biotinylated oligonucleotide (Sigma Aldrich, St. Luis, MO, US) containing the *HNF1A* binding site in the rat albumin promoter; (iii) protein expression in whole cell lysates by immunoblotting;(iv) nuclear localization by indirect immunocytochemistry; and (v) the glucose-stimulated insulin secretion (GSIS) capacity of the variant *HNF1A* in INS1 β-cells were measured using insulin ELISA kit (Mercodia, Sweden). A detailed methodology is described in the Supplementary Material.

2.5 Structural analysis

The human *HNF1A* protein sequence (P20823) was downloaded from the UniProt database. The Consurf server was used to obtain amino acid conservation scores within the orthologous protein family by comparing 150 homologous sequences. For the structure-based stability prediction, the available crystal structure of *HNF1A* in complex with DNA, PDB ID-1IC8 was remodeled with missing residues and was refined using Modeller10v. The refined Wild type (WT) *HNF1A* was considered for stability analysis of *HNF1A* and also the impact of mutants in the *HNF1A*-DNA complex. The structure of mutants was modeled with a WT-*HNF1A* template using Modeller10v, and the refined WT and MT *HNF1A* were subjected to molecular dynamics simulation studies using Gromacs2020 (10.1080/07391102.2021.1965030). Subsequently, PCA and FEL analyses were carried out to determine the near-native conformation, wherein the *HNF1A*-DNA interactions were analyzed using DNAproDB. A detailed methodology is given in the Supplementary Material.

2.6 Statistical analysis

The results of functional analyses of individual variants are presented as mean (in %) ± standard deviation (SD) and normalized to WT *HNF1A* activity (set as 100%), unless otherwise specified. Experiments were carried out on at least 3 independent occasions unless otherwise specified in the figure legends. Statistical differences between individual variants and WT function were analyzed using GraphPad Prism software (version 8.1.1, GraphPad Software, Inc. San Diego, CA, USA) and raw data (i.e., firefly/renilla ratios) and an unpaired 2-tailed t-test based on n=3. A p-value < 0.05 was considered statistically significant.

3 Results

3.1 Clinical and biochemical characteristics of the subjects with *HNF1A* variants

A total of 14 missense *HNF1A* variants identified in 20 clinical MODY patients were included in this study. All the patients were heterozygous for the variants. In three families, we were able to observe the segregation of variants in affected family members, but for other patients, family samples were not available. Pedigrees of the available families are shown in Supplementary Figure 1. All were negative for β-cell autoantibodies such as GAD and ZnT8 antibodies. The mean ± SD of biochemical parameters were as follows: age at onset of diabetes, 21 ± 6.5 years; Body Mass Index (BMI) - 23 ± 4 kg/m2; duration of diabetes, 9.9 ± 6.7 years; Fasting plasma glucose - 181 ± 64 mg/dL; post prandial plasma glucose - 277 ± 97 mg/dL; glycated hemoglobin (HbA1C)- 9.2 ± 2.4%; fasting C-peptide was 0.9 ± 0.4 pmol/L; stimulated C- peptide was 1.5 ± 0.6 pmol/L; total cholesterol - 169 ± 41 mg/dL; triglycerides - 137 ± 82 mg/dL; High Density Lipoprotein (HDL)- cholesterol - 39 ± 8.5 mg/

dL and Low Density Lipoprotein (LDL)- cholesterol - 94 ± 36 mg/dL. Prior to functional genetic investigations, 11 patients were on insulin treatment; one patient was on insulin + metformin; four patients were on insulin + SU; one patient was on metformin alone and three patients were on SU treatment alone before the genetic investigation. Clinical and biochemical parameters are summarized in Table 1.

Among the 14 variants, four variants (p.Lys120Asn, p.Gln125His,p.Ala367Val,p.Asp602Asn) were novel and not reported in the literature, three variants were previously reported by us (3, 24), and the remaining seven variants were reported in other studies (20, 25–29). Of the 14 variants included in this study, six variants reside in DNA binding domain (91-281 a.a), specifically four variants were mapped to POU_S domain (91-181 a.a), one variant was mapped to POU_H domain (203-279 a.a) and one variant reside in the interface between the POUs and POU_H domains of *HNF1A* protein. The other, eight variants were mapped to the transactivation domain (282- 631 a.a) of *HNF1A* protein (Supplementary Figure 2).

3.2 Functional evaluation

3.2.1 Altered transcriptional activity of *HNF1A* variants

In HeLa cells compared to the WT *HNF1A* activity (set as 100%), the measured levels of transcriptional activity (TA) for five (p.Asn127*,p.Val134Ile,p.Arg200Trp and p.Gly292Fs*25)of the 14 variants were significantly lower (<40%) (Figure 1A, Table 2). Three variants (p.Lys120Asn,p.Pro379Ser, and p.Leu611Pro) had TA activity <50%, while two variants (p.Gln125His and p.Thr354Met) had TA activity of 53 and 62% respectively and reduction observed in all these variants were significant. Two variants p.Ala367Val (61%) and p.Asp602Asn (51%) showed a mildly reduced TA. Two other variants (p.Ala301Thr and p.Glu619Lys) demonstrated TA levels comparable to WT *HNF1A* levels (Figure 1A, Table 2). TA was consistently higher for all these variants when using *HNF4A-P2* promoter in INS-1 cells (activity range 32%–137%) (Figure 1B, Table 2) versus rat albumin promoter in HeLa cells. This is most likely due to interference of endogenous *HNF1A* in INS-1 cells (2- to 4-fold higher basal promoter activity).

3.2.2 Effect of variants on DNA- binding activity of *HNF1A* to target DNA sequence

Three variants (p.Asn127*, p.Arg200Trp and p.Arg272His) localized in the DBD and one variant (p.Gly292Fs*25) in TAD demonstrated severely reduced (<40%) activity. All other variants showed normal binding activity comparable to WT (Figure 1C, Table 2).

3.2.3 Effect of variants on *HNF1A* protein expression

Two variants (p.Gly292Fs*25 and p.Ala301Thr) showed significantly reduced protein expression level (<60%); while four variants (p.Gln125His,p.Asn127*,p.Arg200Trp and p.Asp602Asn), demonstrated reduced expression level (61-75%) and were also significant (Figure 1D, Table 2).

3.2.3 Effect of variants on nuclear localization of *HNF1A* protein

All the 14 *HNF1A* variants were assessed for their ability to translocate to the nucleus of the cell in order to regulate their target gene expression. Only four variants showed reduced (~57-67%) nuclear translocation as assessed by indirect immunocytochemistry (Figure 1E, Table 2). Other variants showed normal nuclear translocation.

3.2.4 Effect of variants on insulin secretion

All 14 variants were also assessed for insulin secretion using GSIS. Under basal conditions (2.8mM glucose), these variants produced insulin in the range of 3-15µg/L of insulin and under stimulated conditions using 16.7mM glucose they produced 1-45µg/L of insulin. When they were treated with 100µM glibenclamide (GBC), the stimulated insulin secretion was enhanced ranging from 8-48µg/L in all the 14 variants tested (Figure 1F, Table 2).

3.3 Structural evaluation

Structural analysis was performed for variants found in DNA binding domain. These variants were mapped onto the crystal structure of *HNF1A* protein (PDB ID: 1IC8). Thereby, all the missense variants, namely p.Lys120Asn, p.Gln125His, p.Val134Ile, p.Arg200Trp, and p.Arg272His, were subjected to the following predictions such as sequence and structural-based stability prediction followed by molecular dynamics (MD).

Sequence-based stability study revealed that the *HNF1A* structure is destabilized by the variants p.Lys120Asn, p.Gln125His, p.Arg200Trp, and p.Arg272His, but not by the variant p.Val134Ile. The crystal structure of *HNF1A* in association with DNA (PDB ID-1IC8), was further modified with missing residues and refined using Modeller10v for the structure-based stability prediction (Figure 2A). According to structure-based prediction, the *HNF1A* variants **p.Lys120Asn, p.Arg200Trp, and p.Arg272His** were shown to have a larger destabilizing impact and more molecular flexibility than the other variants. Among these variants, the p.Arg200Trp variant has a higher destabilizing impact. Variants p.Gln125His and p.Val134Ile had the least destabilizing impact (Figures 2B–K). Since the three variants p.Lys120Asn, p.Arg200Trp and p.Arg272His, showed higher destabilizing effects they were chosen for the MD study.

3.3.1 Molecular dynamics stability analysis of the wild and mutant complexes

The WT-*HNF1A* template was used to simulate the structures of the mutants p.Lys120Asn, p.Arg200Trp, and p.Arg272His. The revised WT and MT *HNF1A* were then submitted to MD simulation investigations using Gromacs2020. When the complexes' MD trajectories were compared to the WT, the variant p.Arg272His showed higher divergence than the variants p.Lys120Asn and p.Arg200Trp in the initial period of simulation. However, variant p.Lys120Asn showed more deviations than

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TABLE 1 Clinical and biochemical workup of subjects with *HNF1A* gene variants.

S. No	Patient ID	Gender	Variant	Age at onset (Years)	Duration of Diabetes (Years)	BMI (Kg/m2)	Fasting plasma glucose (mg/dl)	Post prandial plasma glucose (mg/dl)	HbA1C (%)	Fasting C-peptide (pmol/l)	Stimulated-C-peptide (pmol/l)	Total cholesterol (mg/dl)	Triglycerides (mg/dl)	HDL (mg/dl)	LDL (mg/dl)
1	M-026	F	p.Lys120Asn	14	3.7	19.1	188	315	7.1	0.7	1.1	127	61	33	82
2	M-027	M	p.Gln125His	26	6.3	24	134	248	6.9	1	2.2	150	167	32	85
3	M-028	F	p.Asn127Del	14.9	18.1	19.1	277	414	9.5	0.6	0.8	177	134	47	101
4	M-124	M	p.Val134Ile	26.7	6.3	21.9	194	390	9.8	0.5	0.8	136	174	27	94
5	M-125	M	p.Arg200Trp	22.8	16.1	17.9	161	280	8.3	0.5	1.2	152	84	47	88
6	M-126	F	p.Arg200Trp	11	1	23.2	114	171	–	0.9	–	-	-	-	-
7	M-129	F	p.Arg272His	26	8	26.9	106	204	6.4	1.2	2	250	71	45	49
8	M-130	F	p.Arg272His	23	5	23	125	220	6.9	1	2.3	191	209	28	121
9	M-131	F	p.Gly292fs*25	19.1	13	17.3	204	197	10.8	1.1	2	211	176	44	132
10	M-035	F	p.Gly292fs*25	11	4	18.6	127	225	8.7	0.9	1.5	153	114	59	98
11	M-132	M	p.Ala301Thr	28	19	-	114	155	7.3	–	–	193	136	47	125
12	M-133	M	p.Thr354Met	24.8	5	16.2	159	243	6.9	0.7	1.3	125	77	39	71
13	M-138	F	p.Ala367Val	11.6	5	24.1	219	291	11	1	1.6	138	65	43	82
14	M-134	M	p.Pro379Ser	26	6.8	24	268	310	11.4	–	–	270	150	31	209
15	M-135	F	p.Pro379Ser	23	3	26.3	250	310	11.2	2.16	–	145	95	41	85
16	M-036	M	p.Pro379Ser	24	10	27.6	305	521	15.4	0.2	0.3	187	439	37	40
17	M-136	F	p.Pro379Ser	14	–	21.2	289	431	12.7	0.56	1.31	145	95	41	85
18	M-139	F	p.Asp602Asn	14	5	20	159	280	9	2	2.6	195	110	40	70
19	M-137	M	p.Leu611Pro	28.8	18.2	31.6	108	147	6	1.1	3	154	95	30	105
20	M-040	M	p.Glu619Lys	32	27	26.3	134	191	9.5	0.7	1.4	117	160	25	60

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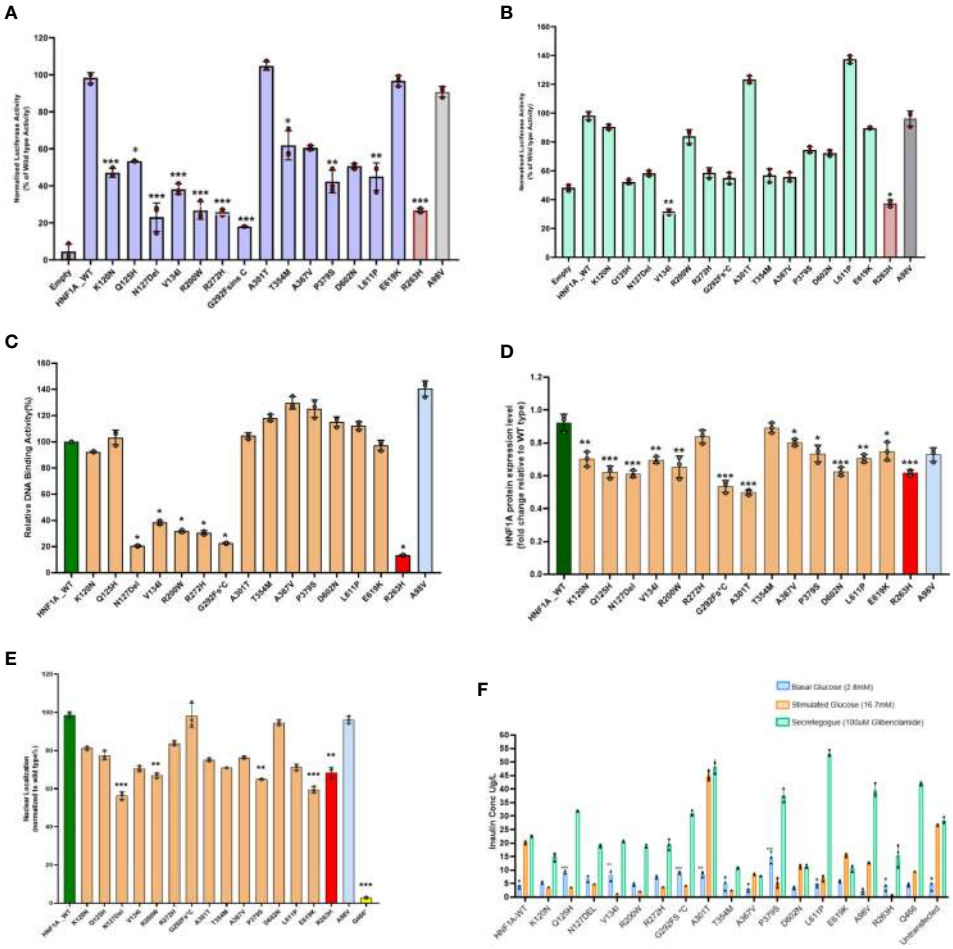

FIGURE 1
Summary of the data obtained from functional studies. **(A)** Transcriptional activity of the *HNF1A* protein variants in HeLa cells; **(B)** Transcriptional activity of the *HNF1A* protein variants in Ins1 cells using HNF4A P2 promoter; **(C)** Assessment of the DNA binding ability of the *HNF1A* protein variants; **(D)** Protein expression levels of the *HNF1A* protein variants in HeLa cells; **(E)** Nuclear localization of the *HNF1A* protein variants in HeLa cells; **(F)** Variant effect on Glucose Stimulated Insulin Secretion. Red bar indicates MODY 3 control variant; Grey bar indicates type 2 diabetes risk variant; Yellow bar indicates variant with poor nuclear translocation effect in *HNF1A* gene. Each bar represents the mean of three independent experiments (n=3) ± SD. P-values were obtained by un-paired student t-test. **** indicates p value <0.001; ** indicates p value <0.01; * indicates p value <0.05.*

p.Arg272His during the last 20 ns of the root mean square deviation (RMSD) plot, a numerical measurement representing the difference between WT and variant protein structures (Figure 2L). The root mean square fluctuation (RMSF) plot, is a calculation of individual residue flexibility, or how much a particular residue moves (fluctuates) during a simulation (Figure 2M), and this showed that residues that interact with DNA were found to have larger deviations in all of the complexes; in particular, residues 179 and 180 of the p.Arg272His variant showed higher deviations of 0.9 nm and 192-193 of the p.Arg272His variant showed higher fluctuations of about 1 nm among the complexes. When compared to WT, the variants p.Lys120Asn and p.Arg272His lost their contact with DNA at the residue level, and their total interactions with DNA also decreased (Figures 2N, O). However, the variant p.Arg200Trp had an increased frequency of interactions with DNA and a greater accessible surface area of all buried solvents (Figures 2N, O). Particularly, the variant residue Trp200 interacts with the minor groove of DNA. From these results, it was revealed that variants p.Lys120Asn and p.Arg272His had lost their interaction with DNA resulting in structural defects.

3.4 Reinterpretation of *HNF1A* variants based on molecular characterization

Pathogenic *HNF1A* variants causing *HNF1A*-MODY are often characterized by significantly decreased TA, poor DNA binding, impaired nuclear targeting, and/or lower protein expression levels in the range of ~20-35% when compared to WT (100%) (19, 21, 30–33). In this study, the cut-off considerations were set at a slightly different level compared to the previous study by Althari et al. (31). Being a more distilled cohort of clinically proven MODY patients, the cut-off of TA<40% was used for pathogenic variants, and TA activity between 40-60% was used for likely pathogenic variants. In addition to this, DNA binding activity, GSIS, and clinical course were considered for ascribing pathogenic and likely pathogenic variants. Therefore, over and above the ACMG/AMP guidelines, the functional and clinical work such as the response to SU have been considered together to re-interpret the variants.

Variants p.Gly292Fs*25 and p.Asn127* were interpreted as pathogenic variants since they have low TA activity along with the

TABLE 2 Summary of the functional studies of the *HNF1A* variants identified in Indian MODY subjects.

	S.No	Amino acid change at protein level	Nucleotide change at c.DNA level	Functional Study								Structure Prediction		
				Transactivation Assay (% WT)		DNA Binding Activity (% WT)	Protein Expression (% WT)	Nuclear Localisation (% WT)	GSIS (Insulin Levels)			Sequence Based Prediction	Structure Based prediction	Molecular Dynamics
				HeLa	Ins 1				Basal	Stimulated	On adding 100μM GBC			
DNA Binding Domain	1	p.K120N	c.360G>C	47	90	92	76	81	5	4	15	Destabilization effect	Higher Destabilization effect	Defect
	2	p.Q125H	c.375G>C	53	52	103	67	77	9	4	32	Destabilization effect	Least Destabilization effect	–
	3	p.N127del	c.377_379delACA	23	58	21	66	57	7	5	19	–	–	–
	4	p.V134I	c.400G>A	38	32	38	75	71	8	1	21	No defect	Least Destabilization effect	–
	5	p.R200W	c.598C>T	27	84	32	71	67	5	2	19	Destabilization effect	Higher Destabilization effect	No defect
	6	p.R272H	c.815G>A	26	59	31	91	84	7	4	19	Destabilization effect	Higher Destabilization effect	Defect
Transactivation Domain	7	p.G292fs*25	c.872-873dupC	18	55	23	58	98	9	4	31	–	–	–
	8	p.A301T	c.901G>A	105	123	105	54	75	8	45	48	–	–	–
	9	p.T354M	c.1061C>T	62	57	118	97	71	5	2	11	–	–	–
	10	p.A367V	c.1100C>T	61	56	130	87	76	3	8	8	–	–	–
	11	p.P379S	c.1135C>T	42	75	125	80	65	15	5	37	–	–	–
	12	p.D602N	c.1804G>A	51	72	115	68	95	3	11	11	–	–	–
	13	p.L611P	c.1832T>C	45	137	112	76	71	5	7	25	–	–	–
	14	p.E619K	c.1855G>A	97	90	97	81	60	6	16	11	–	–	–
	15	p.Arg263His	c.788G>A	27	37	13	67	69	4	1	15	–	–	–
	16	p.Ala98Val	c.293C>T	91	96	141	76	96	2	13	26	–	–	–
	17	p. Gln466*	c.1396 C>T	_	_	_	_	7	_	_	_	–	–	–

Shaded in grey are used as control for the functional assay.

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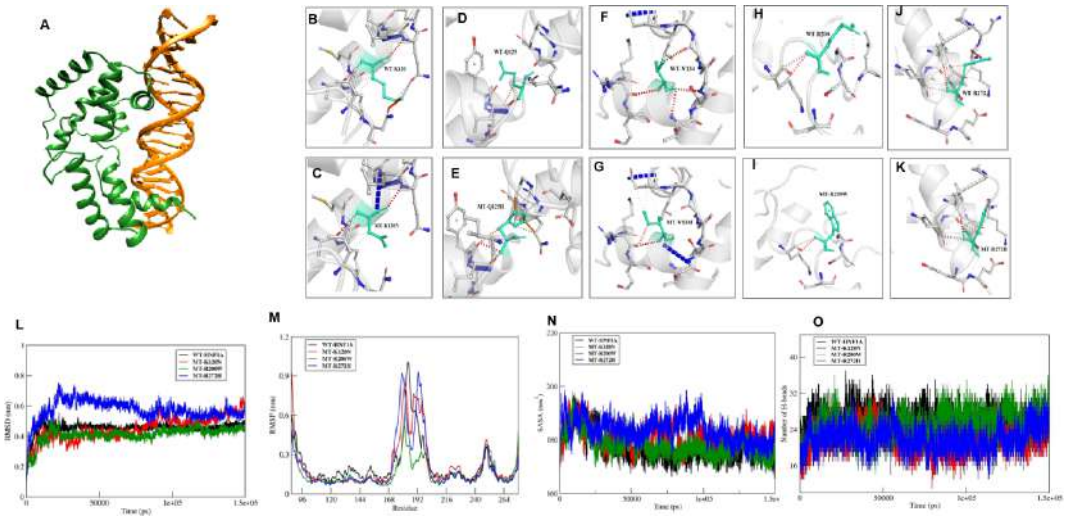

FIGURE 2
In Sillico structural prediction of wild type and variant *HNF1A* protein. **(A)** Remodelled and Refined Wild-type (WT) *HNF1A*-DNA complex; **B-K**) Prediction of Interatomic Interactions of the Wild and mutant forms of *HNF1A* variants, where the Wild-type and mutant residues are coloured in light-green and are also represented as sticks alongside the surrounding residues which are involved in any type of interactions; **(L-O)** Molecular dynamics simulation analysis of the wild and MT forms of *HNF1A* complexes **(L)** RMSD plot **(M)** RMSF plot **(N)** Solvent accessible surface area plot; **(O)** Number of inter hydrogen bonds maintained throughout the MD production run within *HNF1A* and DNA.

reduced DNA binding activity and defect in insulin secretion.p.Arg272His was reinterpreted as a pathogenic variant from their initial interpretation. Seven variants (p.Lys120Asn, p.Gln125His, p.Val134Ile, p.Arg200Trp, p.Thr354Met, p.Pro379Ser, and p.Leu611Pro) were reclassified as likely pathogenic variants from VUS. Three variants (p.Ala367Val, p.Asp602Asn, and p.Glu619Lys) remained VUS after reinterpretation whereas variant p.Ala301Thr was reinterpreted as benign from VUS (Figure 3, Table 3).

3.5 Clinical follow-up of the patients with *HNF1A* variants

Variants designated as pathogenic/likely pathogenic based on functional assessment were investigated for clinical actionability by collecting the follow-up details of the patients over a period of time. The patient (M-026) with variant p.Lys120Asn has been switched from insulin to two doses of SU (glimepiride) along with metformin per day. The patient M-027 with the mutation p.Gln125His (likely pathogenic variant) developed diabetes at the age of 25.7 years and had diabetes for 7 years. Before genetic testing, the patient was treated with insulin and oral hypoglycemic agents (OHA). As a result of genetic studies, the patient was transferred from insulin to two doses of gliclazide per day. His HbA1C levels dropped from 9.6% to 6.4% after his therapy was changed.

Patient M-028, who carries the pathogenic variant p.Asn127*, is diagnosed with diabetes at the age of 14.9 years, with a duration of 15.6 years (Figure 4). The patient was on OHA for around two years before being started on insulin. She is currently on insulin and SU therapy since her β cell reserve was low (CPF-0.6 and CPS-0.9) and she started to develop microvascular and macrovascular complications. Patient M-124 harboring the variant p.Val134Ile

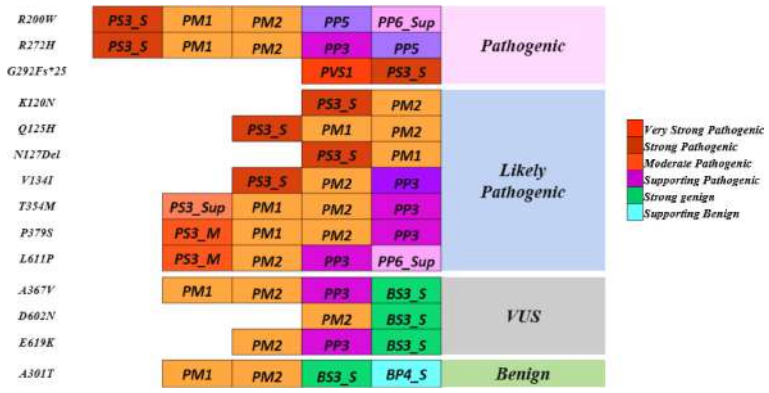

FIGURE 3
Reinterpretation of *HNF1A* variants using molecular characterization.

TABLE 3 Summary of re-interpretation of *HNF1A* gene variants and their clinical actionability, identified in Indian MODY patients based on molecular characterization.

	S.No	Amino acid change at protein level	Nucleotide change at c.DNAlevel	Variant Interpretation_ ACMG guidelines 2015		Functional Study								Structure Prediction			Reinterpation Based on functional evidence		Clinical Actionability
						Transactivation Assay (% WT)		DNABinding Activity (% WT)	Protein Expression (% WT)	Nuclear Localisation (% WT)	GSIS (Insulin Levels)			Sequence Based Predic-tion	Structure Based predic-tion	Molecular Dynamics			
				Evidence	Classification	HeLa	Ins 1				Basal	Stimulated	On adding 100μM GBC				Evidence	Classification	
DNA Binding Domain	1	p.K120N	c.360G>C	PM1, PM2	VUS	47	90	92	76	81	5	4	15	Destabilization effect	Higher Destabilization effect	Defect	PS3_Moderate, PP3_Strong	LP	Actionable
	2	p.Q125H	c.375G>C	PM1, PM2	VUS	53	52	103	67	77	9	4	32	Destabilization effect	Least Destabilization effect	–	PS3_Moderate, PP3 and PP6	LP	Actionable
	3	p.N127del	c.377_379delACA	PM1, PM2	VUS	23	58	21	66	57	7	5	19	–	–	–	PS3_Strong	P	Actionable
	4	p.V134I	c.400G>A	PM1, PM2,PP3	VUS	38	32	38	75	71	8	1	21	No defect	Least Destabilization effect	–	PS3_Strong	LP	Actionable
	5	p.R200W	c.598C>T	PM1, PM2,PP5	VUS	27	84	32	71	67	5	2	19	Destabilization effect	Higher Destabilization effect	No defect	PS3_Strong	P	Actionable
	6	p.R272H	c.815G>A	PM1, PM2, PP3, PP5	LP	26	59	31	91	84	7	4	19	Destabilization effect	Higher Destabilization effect	Defect	PS3_Strong	P	Actionable
Transactivation Domain	7	p.G292fs*25	c.872-873dupC	PVS1	LP	18	55	23	58	98	9	4	31	–	–	–	PS3_Strong	P	Actionable
	8	p.A301T	c.901G>A	PM1, PM2	VUS	105	123	105	54	75	8	45	48	–	–	–	BS3_Strong, BP4_Strong	B	_
	9	p.T354M	c.1061C>T	PM1, PM2,PP3	VUS	62	57	118	97	71	5	2	11	–	–	–	PS3_Supporting	LP	Actionable
	10	p.A367V	c.1100C>T	PM1, PM2	VUS	61	56	130	87	76	3	8	8	–	–	–	BS3_Strong, BP4_Strong	VUS	Unresolved
	11	p.P379S	c.1135C>T	PM1, PM2, PM5,PP3	LP	42	75	125	80	65	15	5	37	–	–	–	PS3_Moderate	LP	Actionable
	12	p.D602N	c.1804G>A	PM1, PM2	VUS	51	72	115	68	95	3	11	11	–	–	–	BS3_Strong	VUS	Unresolved
	13	p.L611P	c.1832T>C	PM1, PM2,PP3	VUS	45	137	112	76	71	5	7	25	–	–	–	PS3_Moderate	LP	Actionable
	14	p.E619K	c.1855G>A	PM1, PM2,PP3	VUS	97	90	97	81	60	6	16	11	–	–	–	BS3_Strong	VUS	Unresolved

P, Pathogenic; LP, Likely Pathogenic; B, Benign; VUS, Variant of Uncertain significance.

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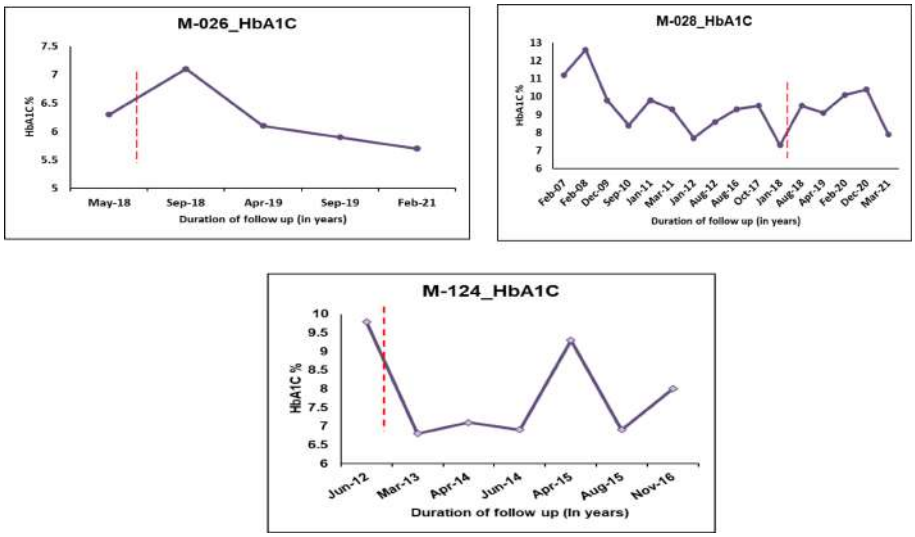

FIGURE 4
HbA1C Trajectories of few *HNF1A* MODY patients after change in treatment based on re-interpretation of the variants. Dotted lines indicate change of treatment.

(Likely pathogenic variant) was diagnosed with diabetes at the age of 26.7 years with diabetes duration of 4 years. Based on functional evidence, patient M-124 with variant p.Val134Ile was transitioned from insulin to a single dose of glipizide per day.

Patient M-126 with the pathogenic variant p.Arg200Trp was switched from insulin to SU. It was advised to continue with SU for patient M-125 who had the same variant. Statins were given for patient M-125 in order to maintain a normal lipid profile. Previous studies have shown two other amino acid changes at the same codon such as p.Arg200Gly and p.Arg200Gln in multiple SU-sensitive *HNF1A*-MODY families (34, 35). The functional effects of these two variants, p.Arg200Gly and p.Arg200Gln, were however not mentioned. All of the patients, including the one from this study, who have the variation in this codon respond to SU. This suggests that the variation is pathogenic and clinically actionable. Patients with pathogenic variant (p.Arg200Trp, p.Arg272His and p.Gly292Fs*25) and likely pathogenic variant (p.Thr354Met and p.Leu611Pro) were also shifted from insulin to SU therapy.

4 Discussion

The comprehension of disease mechanisms is improved by well-established functional investigations on variants, which also offer proof for the pathogenicity of the variants. Studies have demonstrated that functional studies help to clarify the interpretation of *HNF1A*-MODY variants, particularly in the absence of familial segregation or phenotypic data (32).

In this study, we have performed molecular characterization of 14 *HNF1A* variants identified in 20 unrelated individuals from 20 non-consanguineous families among Indian MODY subjects, where the majority of variants have not been reported. Normal transactivation activity of *HNF1A* protein, which depends on the capacity to bind target promoters (DNA) and on an adequate quantity of cellular (nuclear) protein, is necessary for normal *HNF1A* transcription factor function.

Because not all functional tests represent the underlying process and not all variants have the same effects on function (36), we aimed at improving the understanding and interpretation of these findings. Therefore, multiple assays were employed to fully examine the effects of a variant in order to come to a conclusion. These variants were examined utilizing *in vitro* functional pipelines, such as luciferase assays for transactivation, which measure the transcriptional activity of *HNF1A* variants, as well as assays of DNA binding activity, protein expression, and subcellular localization to determine the impact of the variants on the protein function. Additionally, a GSIS assay to examine the impact of these variants on insulin secretion was performed. A distinctive feature of this work is the *in silico* structural analyses to determine if it might identify the variants with functional defects. Since the crystal structure of *HNF1A* is available only for the DNA binding domain, structural investigations were carried out for the missense variants identified only in that region.

A multi-pronged approach using the ACMG guidelines, the functional and structural analyses have been considered together to re-classify these variants. In this work, we focused on the scoring systems and the criteria for re-interpreting the variants. PS3 was assigned when data from well-established *in vitro* functional studies supported a detrimental effect on the gene or gene product; PP3 was assigned when multiple lines of computational evidence and structural prediction supported a detrimental effect on the gene or gene product (conservation, evolutionary, etc.); and BS3 was assigned when well-established *in vitro* functional studies showed no detrimental effect on protein function. In addition, multiple levels of strength, such as strong, moderate, and supporting levels based on functional and structural data were applied to the scoring

approaches employed in this study. Of the 14 variants considered in this study, 1 variant p.Arg272His was interpreted as likely pathogenic, and 11 variants were interpreted as VUS initially based on the ACMG/AMP guidelines. (Figure 3, Table 3).

According to previous studies on the effects of pathogenic *HNF1A*-MODY variants, pathogenic and MODY causal variants impair *HNF1A* activity, DNA binding, and localization (40% compared to WT *HNF1A*) (21, 32), whereas type 2 diabetes risk variants have an impact on *HNF1A* function ranging from 40% -60% compared to WT (30, 31, 33).

Based on the aforementioned cut-offs, many degrees of strength were assigned to each scoring criterion. PS3_Strong scoring criteria were assigned to variants that showed <40% activity than WT activity in at least two functional assays; PS3_Moderate was assigned to variants that showed activity between 40 and 60%; and PS3_Supporting was assigned to variants that showed activity less than 65%. PP3_Strong criterion was assigned when the variant showed defects in all the *in silico* structural prediction analysis. The variant meeting the BS3_Strong criterion had no negative effect on protein function in any of the functional experiments.

The *p.Arg272His* previously interpreted as likely pathogenic was re-interpreted as *pathogenic* based on the evidence *PS3_Strong, PM1, PM2, PP5, and PP3_Strong*. One variant *p.Arg200Trp* interpreted as VUS was re-interpreted as *pathogenic* based on the evidence *PS3_Strong, PM1, PM2, PP3_Supporting, and PP5*. Variant *p.Gly292Fs*25* was interpreted as *pathogenic* based on the evidence *PVS1 and PS3_Strong* and variant *p.Asn127** was interpreted as *likely pathogenic* based on the evidence *PS3_Strong, PM1*. Variants *p.Lys120Asn* and *p.Gln125His* interpreted as VUS was re-interpreted into *likely pathogenic* based on the evidence *PS3_Moderate, PM2, PP3_Strong, and PS3_Moderate, PM2, PP3_Supporting, PP6* respectively. Variant *p.Val134Ile* was re-interpreted into *likely pathogenic* based on evidence *PS3_Strong* and *PM2*. Variant *p.Thr354Met* was re-interpreted as likely pathogenic based on *PS3_Supporting, PM1, PM2, and PP3*. Variant *p.Pro379Ser* was re-interpreted as *likely pathogenic* based on the evidence *PS3_Moderate, PM1, PM2, and PP3*. Variant *p.Leu611Pro* was re-interpreted as *likely pathogenic* based on the evidence *PS3_Moderate, PM2, PP3, and PP6_Supporting*. Variant *p.Ala367Val* remains *VUS* based on the evidence *PM1, PM2, PP3, and BS3_Strong*. Variants *p.Asp602Asn* and *p.Glu619Lys* remain *VUS* based on the evidence *PM2, BS3_Strong and PM2, PP3, and BS3_Strong* respectively. Variant *p.Ala301Thr* was re-interpreted as *benign* based on the evidence *PM1, PM2, BS3_Strong, and BP4_Strong* (Table 3). It is crucial to remember that functional evidence does not always associate a variant to disease outcome; in order to determine clinical actionability, the functional data must be assessed in combination with clinical data (30). It is important to be aware of the fact that both functional and longitudinal clinical follow up are important to establish the clinical actionability of the variants.

Clinical actionability is generally defined as clinically prescribed interventions that are effective for preventing or delaying clinical disease, lowering clinical burden, or improving clinical outcomes in an adult who has not previously received a diagnosis and are specific to the genetic disorder under consideration (37). Based on our results, 4 out of 14 (28.6%) variants were interpreted as pathogenic, 6 variants (42.8%) as likely pathogenic, 3 variants (21.4%) as variants of uncertain significance, and 1 variant (7.14%) as a benign variant. Patients with the ten P/LP variants were able to successfully switch from insulin to SU and sustain good glycemic control, thus making these variants clinically actionable (Table 3).

We performed 3D structural analysis to check whether *in-silico* analysis corroborated with functional investigations in identifying the pathogenic variants and also to have a structural understanding of the variant *HNF1A* proteins. Our *in-silico* analysis showed that variants p.Gln125His, p.Val134Ile have lesser structural defects while variants p.Lys120Asn and p.Arg272His have severe structural defects, and the variant p.Arg200Trp has moderate structural defects. In the case of the p.Val134Ile variant, we found differences between the functional and structural data. Although *in-silico* structural analysis showed that it has a lesser destabilizing effect despite being predicted to be a highly conserved structural residue, our functional data showed that variant p.Val134Ile has a defect in DNA binding thus down-regulating the target genes resulting in reduced insulin secretion (Table 2). Moreover, the patient follow-up also showed that the patient (M-124) responded well to treatment change to SU, making this variant a clinically actionable one (Figure 4).

Our study has a few limitations. Since we could not obtain family samples for many patients, we were unable to conduct family co-segregation studies. In some patients, we did not have adequate clinical data.

In summary, this paper exemplifies the importance of performing molecular characterization after genetic testing, since the understanding of the functional basis of genotypes helps in understanding the phenotype which could lead to changes in clinical treatment for monogenic disorders like MODY. Our findings are the first to show the need of using additive scores during molecular characterization for accurate pathogenicity evaluations of *HNF1A* variants in precision medicine. Furthermore, it is also one of the first to introduce structural understanding to functional implications. The study has led to the delineation of the VUS into pathogenic and disease-causing MODY variants, from non-pathogenic variants. Patients with most pathogenic *HNF1A* variants benefit from OHA treatment; hence, this would assist clinicians in determining the best course of action for patients. While the combination of functional and structural-based approaches may lead to increased certainty in variant–phenotype correlation in a research setting, a functional understanding of the variants helps in precision diagnosis and treatment in a monogenic disorder such as MODY.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Ethics statement

The studies involving human participants were reviewed and approved by Institutional ethics committee, MDRF. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

VR and BK designed and implemented the functional study. BK analyzed the data and wrote the manuscript. SR designed and performed the structural analysis. UV and NH analyzed the structural data. SG performed segregation analysis. VM collected the clinical data and analyzed the manuscript. VR analyzed all data and corrected the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1177268/full#supplementary-material

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ORIGINAL ARTICLE

Genotype-phenotype correlation of K_{ATP} channel gene defects causing permanent neonatal diabetes in Indian patients

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Abstract

Background: There are very few reports pertaining to Indian patients with neonatal diabetes mellitus (NDM). Activating or gain of function mutations of K_{ATP} channel genes namely *KCNJ11* and *ABCC8* are most predominant cause of permanent neonatal diabetes mellitus (PNDM).

Objectives: To identify the genotype-phenotype correlation of K_{ATP} channel gene defects in a large series of (n = 181) Indian PNDM patients.

Methods: Direct sequencing of all exons of *KCNJ11* and *ABCC8* genes in all 181 patients with PNDM were performed. Clinical and biochemical data were collected.

Results: We have identified the molecular basis of K_{ATP}-NDM in 39 out of 181 patients (22%). Of these, 20 had *KCNJ11* mutations and 19 had *ABCC8* mutations, thus comprising 51% of *KCNJ11* and 49% of *ABCC8*. There were four novel mutations (D1128Tfs*16, Y1287C, S1422T, and H1537R) in *ABCC8* gene. Three patients with *KCNJ11* mutations had developmental delay with DEND syndrome. In patients with *ABCC8* mutations developmental delay was seen in seven out of 19 (36.8%). Of this, three patients (15.7%) had DEND phenotype and four (21%) had iDEND. Of the 39 patients, 33 (84%) patients were shifted to sulfonylurea therapy (glibenclamide). Of this, 19(57.5%) patients harbored *KCNJ11* mutations and 14(42.1%) *ABCC8* mutations.

Conclusions: This is the first largest study in NDM patients in India demonstrating the importance of K_{ATP} channel gene mutation screening in PNDM and efficacy of glibenclamide for Indian patients with K_{ATP}-PNDM. The success rate of transfer is more in patients with *KCNJ11* mutations compared with those with *ABCC8* mutations.

KEYWORDS

ABCC8 gene, activating mutation, *KCNJ11* gene, permanent neonatal diabetes mellitus in India, sulfonylurea

1 | INTRODUCTION

Neonatal diabetes mellitus (NDM) is defined as diabetes mellitus that develops during the first 6-9 months of life.[1,2] It is a rare disorder with an incidence of 1 in 100 000-200 000 live births[3] NDM can be a transient form characterized by remission of diabetes within several months and relapse to diabetes during adolescence or early adulthood or it can be permanent neonatal diabetes (PNDM) which requires lifelong treatment for controlling the blood glucose of the patient from the time of diagnosis.[2]

ATP-sensitive K^+ (K_{ATP}) channels are present in the membranes of many organs and cell types such as pancreas, skeletal muscle, brain and heart and play a critical role in linking metabolism to electrical activity of the cell.[4,5] In pancreatic β cells, K_{ATP} channels couple the glucose metabolism with electrical activity for the production of insulin to maintain the glucose homeostasis. K_{ATP} channels are formed by an unique hetero-octameric combination of two dissimilar proteins: the Kir6 (Potassium Inward Rectifier 6) which is a member of the Kir channel family; and the sulphonylurea receptor (SUR),which is a member of the ATP-binding cassette (ABC) protein family. Pancreatic K_{ATP} channel is formed by Kir 6.2 and SUR1 protein encoded by *KCNJ11* and *ABCC8* gene respectively. K_{ATP} channels are positively regulated by binding of cytoplasmic ATP and ADP to -Kir6.2 subunit and negatively regulated by binding of nucleotide to -SUR1 subunit in the glucose induced insulin secretion pathway. Gain of function or activating mutations in K_{ATP} channel results in a pathophysiological condition called neonatal diabetes mellitus (NDM) and Loss of function or inactivating mutations result in a condition called congenital hyperinsulism (CHI).[6,7] Most of the gain of function mutations cause only NDM, while some mutations cause DEND syndrome which is a severe pathological condition of neonatal diabetes accompanied by developmental delay, muscle weakness, and epilepsy.[8] Less severely affected patients show intermediate DEND (iDEND) syndrome characterized by mild to moderate developmental delay without epilepsy.[2]

The most common cause of PNDM are activating mutations in the K_{ATP} channel genes namely, *KCNJ11* and *ABCC8* genes followed by *INS*. Mutations in other genes, including *IPF*, *GCK*, and *FOXP3* have also been reported to cause PNDM but these are very rare. To date there are about 200 different mutations in K_{ATP} channel genes reported in Human Gene Mutation Database (HGMD) to cause various forms of NDM and DEND syndrome. Clinical severity of this pathophysiological condition is in the following order: DEND > iDEND > isolated PNDM > TNDM or late-onset diabetes. Functional studies have demonstrated that the residual channel current roughly correlates with the clinical severity in the same order.[9,10] Most of the patients with activating or gain of function mutations in K_{ATP} mutations are able to switch from insulin injection to oral hypoglycemic agents such as sulfonylureas.[11] Sulfonylureas bind to the SUR1 subunit of K_{ATP} channel to reduce the open probability (P_o), and this leads to increased insulin secretion from the β cells.[15] Patients with K_{ATP} channel mutations are managed well with sulphonylurea than with insulin and even patients with neurological complications are greatly benefited with sulphonylurea.[12,13] Understanding the genetic basis of NDM in general has greatly facilitated the correct diagnosis and treatment of this disease.

Although data on genotype-phenotype correlation and the effects of sulfonylurea are accumulating, there have been very few reports from the Indian population on neonatal diabetes. We and others have published on a small number of patients of NDM in addition to single case reports.[14-18] Since mutations of K_{ATP} channel genes are the most predominant cause of PNDM, here we report and describe the genetic basis of K_{ATP} channel genes in a larger series of 181 Indian children with PNDM along with their clinical profile and management strategies. This is the first ever study on the genetic etiology of large cohort of Indian children.

2 | METHODS

2.1 | Study subjects

The study group comprised of children with neonatal diabetes referred to Madras Diabetes Research Foundation for genetic testing. For all cases, informed consent was obtained from the parents. All probands included in the study were negative for pancreatic autoimmune antibodies (glutamic acid decarboxylase autoantibodies [GAD] and islet antigen 2 autoantibodies [IA2]). A total of 181 children who had diabetes as defined by random plasma glucose greater than 200 mg/dL on more than one occasion and with the onset of diabetes on or before 6 months of age were included and were classified as "Permanent neonatal diabetes mellitus" (PNDM). None of the patients had remission of diabetes as was evident from the follow up data. We also included 100 normal glucose tolerant subjects for the study (fasting value <100 mg/dL and 2 hours value <140 mg/dL). Blood samples from parents were collected wherever possible to check for the co-segregation of the mutations identified. The study was approved by the local institutional ethical committee of the Madras Diabetes Research Foundation (MDRF), India. Written informed consent was obtained from all the parents or guardians of the children before the study. The reported investigations have been carried out in accordance with the Declaration of Helsinki principles as revised in 2000.

2.2 | Genetic analysis

DNA was isolated from whole blood by phenol chloroform method. Direct sequencing was carried out on ABI 3500 Genetic Analyzer (Applied Bio systems, Foster City, California) using the Big Dye terminator V3.1 chemistry and the sequences were compared with the public data bases. Published primer sequences were used to amplify the DNA for *KCNJ11* and *ABCC8* genes. In addition to sequencing of patients, we also sequenced 100 normal glucose tolerant subjects

(fasting value <100 mg/dL and 2 hours value <140 mg/dL) to check for the absence and presence of mutation in them.

2.3 | Bioinformatic analysis

Clinically relevant mutations were annotated using published variants in literature and a set of diseases databases such as gnomAD, ClinVar, HGMD, and ACMG clinical guidelines. Common variants were filtered based on allele frequency in 1000 Genome and Exome Aggregation Consortium (ExAC).

3 | RESULTS

We set out to identify the molecular correlation of K_{ATP} channel gene defects in PNDM in a large series of 181 patients who were referred to MDRF for genetic screening and analysis by Pediatricians and Neonatologist from all over India. The period under study was between 2014 and 2019.

We have identified and described the genotype-phenotype correlation of K_{ATP} -NDM in 39 out of 181 patients (comprising 22%). All these patients were PNDM. Of the 39 patients, 20 had mutations in *KCNJ11* gene and 19 in *ABCC8* gene, constituting 51% in *KCNJ11* and 49% in *ABCC8*. The percentage of consanguineous families in the total patients was 18% and in many patients with a heterozygous mutation born from consanguineous parents, the mutation was spontaneous (eg, IND-10, IND 21, and IND 38).

3.1 | PNDM due to mutations in *KCNJ11* gene

Table 1 shows the features of all 39 K_{ATP}-PNDM patients, drawing a comparison between those with *ABCC8* mutation and those with *KCNJ11* mutation. Statistically there were no significant differences in gender distribution, gestational age, birth weight, age at onset, plasma glucose, or DKA at presentation.

Table 2 summarizes the mutations and clinical presentation of each *KCNJ11*-PNDM patient. In 20 patients with *KCNJ11*-NDM we identified nine different mutations. All the mutations were previously known. Five patients harbored R201C, six harbored R201H, two harbored R50Q, two had V59M, and remaining five mutations were identified in one patient each. All the mutations were heterozygous in nature.

Figure 1A,B show the schematic representation of mutations in *KCNJ11* and *ABCC8* gene. Figure 2 shows the pedigrees of patient with *KCNJ11* mutations. Of the 20 patients with mutation, two were born of consanguious marriage (IND-10 and IND-13). About 14 of the mutations were *de novo* in origin. In patient IND-13 the mutation could be traced to the father who was a non-diabetic. In patients IND-1, IND-17, IND-18, and IND-20 parents' samples were not available for testing. In patient IND-14, only one parent sample was available and hence inheritance could not be tested.

As seen in previous studies, the substitution of arginine by cysteine (c.602C>T, p.R201C) and substitution of arginine by histidine at codon 201 (c.601G>A, p.R201H) were the most common, with the former in five and latter in six patients. Interestingly the random blood sugar (RBS) was greater in those harboring R201C compared with those with R201H (*P* value:.0536). Three patients (IND-3, IND-4, and IND-5) showed characteristic features of DEND such as developmental delay, epileptic seizures along with diabetes, of which two (IND-3 and IND-4) had V59M mutation and one (IND-5) had V64M mutation.

3.2 | PNDM due to mutations in *ABCC8* gene

Table 3 summarizes the mutations and clinical presentation of each *ABCC8*-PNDM patient. In 19 patients with *ABCC8*-NDM, we identified 16 different mutations. Of these 4 mutations were novel (D1128Tfs*16, Y1287C, S1422T, and H1537R) and 12 were previously known to cause NDM. The reported mutations found in the study are R1182W in three patients, F132L in two patients, V215I in two patients, L225P in two patients, and all other mutations were found in only one patient. Mutations such as A1263V (IND-35) and D1128T fs*16 (IND-36) were found in homozygous state. One patient was a compound heterozygote for G70R and H1537R mutations, while others were heterozygous in nature.

Figure 3 shows the pedigrees of patients with *ABCC8* gene mutations. Of the 19 patients with mutation four were born out of consanguious marriage (IND-21, IND-35, IND-36, and IND-39). Ten of nineteen patients have arisen de novo (IND-21, IND-22, IND-25, IND-27, IND-28, IND-29, IND-31, IND-33, IND-34, and IND-39). Mutation could be traced back to mother in three families (IND-24, IND-30, and IND-35), and to father in one family (IND-26). Paternal samples were unavailable for IND-23 and IND-32 patients. Amongst the remaining three, two (IND-35 and IND-36) harbored mutations in

TABLE 1 Clinical features of 39 patients with PNDM

	Patients with KCNJ11 mutation (n = 20)	Patients with ABCC8 mutation (n = 19)	*P* value
Gender (male/female)	(8/12)	(12/8)	—
Gestational age (weeks, mean)	38.6	38.3	0.7
Birth weight (g, mean)	2562.5	2517	0.72
Age at onset (months, mean ± SD)	3.2 ± 1.7	3 ± 2	0.73
Plasma glucose (mg/dL [mmol/L], mean)	500 (27.7)	417 (23)	0.1
DKA at presentation (yes/no), %	(13/3), 81% (n = 16)	(5/8), 62% (n = 13)	—
Sulfonylurea response (%)	57.5	42.1	—

TABLE 2 Clinical characteristics of patients with *KCNJ11* gene mutation

Patients	*IND 1*	*IND 2*	*IND 3*	*IND 4*	*IND 5*	*IND 6*	*IND 7*	*IND 8*	*IND 9*	*IND 10*
Phenotype	PNDM	PNDM	PNDM	PNDM	PNDM	PNDM	PNDM	PNDM	PNDM	PNDM
Gender	F	M	M	M	F	M	F	M	F	F
Current age (years)	5.4	1.10	4.8	0.10	1.10	2.6	5.9	5.9	5.2	3
Age at diagnosis (months)	5.5	1	3	2	6	2	2	4	5	2
Birth weight (g)	3000	2000	2800	3000	2000	3000	2200	2700	2600	2250
Birth weight (centiles)	—	—	90	97	25	97	10	—	—	50
RBS (mg/dL)	485	540	382	500	502	522	1100	400	746	517
Consanguinity	No	No	No	No	No	No	No	No	No	Yes
Developmental delay	No	No	Yes	Yes	Yes	No	No	No	No	No
Mutations										
cDNA	c.149G>A	c.149G>A	c.175G>A	c.175G>A	c.190G>A	c.510G>C	c.601C>T	c.601C>T	c.601C>T	c.601C>T
Zygosity	Hetero	Hetero	Hetero	Hetero	Hetero	Hetero	Hetero	Hetero	Hetero	Hetero
Protein	p.R50Q	p.R50Q	p.V59M	p.V59M	p.V64M	p.K170N	p.R201C	p.R201C	p.R201C	p.R201C
Novel	No	No	No	No	No	No	No	No	No	No
DKA at presentation	—	—	No	Yes	Yes	Yes	Yes	—	—	Yes
Insulin therapy after clinical diagnosis (U/kg/day)	3	9	3	9	4	3	5	10	5	1
SU treatment	+	+	+	+	+	+	+	+	+	+
SU treatment initiated (years)	1.1	0.4	0.5	0.5	1	0.4	0.4	0.8	0.6	0.3
Response	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
Other medication	No	No	No	No	Insulin + valproic acid	No	No	No	No	No
RBS after shifting (mg/dL)	132	126	110	120	142	115	125	121	117	121
RBS during recent follow up (mg/dL)	108	110	115	118	130	117	113	109	105	110
Follow up (years)	4.6	1.6	4.3	0.2	0.7	2.7	5.6	5.1	4.9	2.9

TABLE 2 Clinical characteristics of patients with *KCNJ11* gene mutation (Contd…)

Patients	*IND 11*	*IND 12*	*IND 13*	*IND 14*	*IND 15*	*IND 16*	*IND 17*	*IND 18*	*IND 19*	*IND 20*
Phenotype	PNDM	PNDM	PNDM	PNDM	PNDM	PNDM	PNDM	PNDM	PNDM	PNDM
Gender	M	F	F	F	F	M	M	F	F	F
Current age (years)	0.4	6	3.10	3.2	7.4	3.6	0.5	5.10	1.4	4.11
Age at diagnosis (months)	1.5	3	6	5	4	3	1	5	2	1
Birth weight (g)	2750	2000	2750	2500	2700	3000	2600	2500	2900	2000
Birth weight (centiles)	75	25	75	50	75	95	90	50	97	25
RBS (mg/dl)	680	399	524	600	225	450	220	480	364	371
Consanguinity	No	No	Yes	No	No	No	No	No	No	No
Developmental delay	No	No	No	No	No	No	No	No	No	No
Mutations										
cDNA	c.601C>T	c.602G>A	c.602G>A	c.602G>A	c.602G>A	c.602G>A	c.602G>A	c.679G>A	c.754G>T	c.1001G>T
Zygosity	Hetero	Hetero	Hetero	Hetero	Hetero	Hetero	Hetero	Hetero	Hetero	Hetero
Protein	p.R201C	p.R201H	p.R201H	p.R201H	p.R201H	p.R201H	p.R201H	p.E227K	p.V252L	p.G334V
Novel	No	No	No	No	No	No	No	No	No	No
DKA at presentation	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	No
Insulin therapy after clinical diagnosis (U/kg/day)	3	7	3	2	7	2	4	5	5	11
SU treatment	+	+	+	+	+	+	+	+	+	+
SU treatment initiated (years)	0.4	1	0.9	0.6	7	0.6	0.2	0.5	0.4	0.2
Response	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Other medication	No	No	No	No	No	No	No	No	No	No
RBS after shifting (mg/dL)	128	124	158	177	135	124	132	125	132	121
RBS during recent follow up(mg/dL)	113	117	121	126	122	105	117	105	117	100
Follow up (years)	0.4	5	3.1	2.10	1.8	3	0.3	5.4	1	4.9

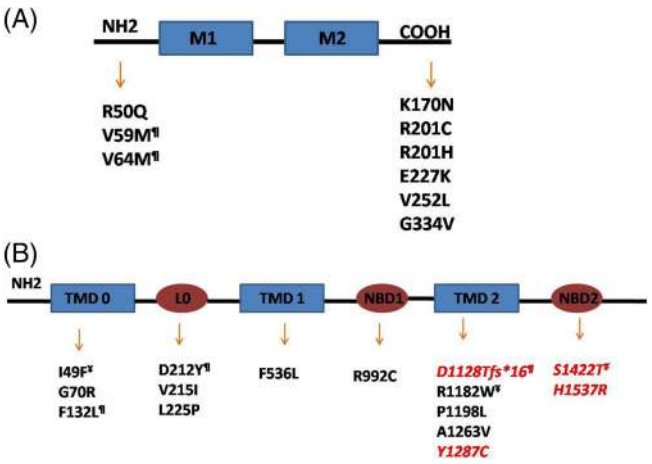

FIGURE 1 A, Schematic representation of functional domains of *KCNJ11* gene with mutations. B, Schematic representation of functional domains of *ABCC8* gene with mutations

homozygous condition, the other patient IND-39 had compound heterozygous condition inheriting each allele from each parent. IND-35 and IND-36 patients showed recessive mode of inheritance.

Developmental delay was seen in seven patients out of 19 (36.8%). Of this, three patients (15.7%) had DEND phenotype and four (21%) are classified as iDEND.

3.3 | Sulfonylurea therapy

After the molecular diagnosis and analysis of K_{ATP} channel gene mutations in the 39 patients, 33 (84%) patients were shifted to sulfonylurea therapy (glibenclamide). Of this, 19 (57.5%) harbored mutations in *KCNJ11* and 14 (42.4%) harbored mutations in *ABCC8* gene (Table 1). The patient with DEND harboring *KCNJ11* mutation (V59M) has been shifted to SU (Glibenclamide) with a dosage of 0.7 mg/kg/day, while another patient with DEND in *KCNJ11* mutation (V64M) could not be shifted to SU and so she continued to have insulin treatment with antiepileptic drug (valproic acid). Among patients with DEND in *ABCC8* mutation; two (D212Y and D1128Tfs*16) out of three have been shifted to SU (Glibenclamide) with a dosage of 0.8 and 0.9 mg/kg/day, respectively, along with antiepileptic drug while one patient (F132L) with milder DEND continues to be on insulin with antiepileptic drug. Four patients with *ABCC8* mutations (I49F, F132L, R1182W, and S1422T) have iDEND, two (I49F and R1182W) of whom have been shifted to SU while other two (F132L and S1422T) did not respond to SU therapy and hence continue to be on insulin therapy. We found the success rate of transfer to be more in patients with *KCNJ11* compared with those with *ABCC8* mutations.

4 | DISCUSSION

This is the largest study describing the spectrum of mutations in K_{ATP} channel-PNDM in Indian patients. Activating or gain of function mutations in *KCNJ11* and *ABCC8* genes are known to be the predominant cause of PNDM in various populations in the world.[3,8-10] Hence these two genes were screened for genetic defects in the present study.

In this study, we identified the molecular defects in 22% of the PNDM patients. Of these, *KCNJ11* and *ABCC8* mutations were in near equal proportion. This is in contrast to the other populations such as Caucasians and Japanese where the *KCNJ11* mutation is predominant over the *ABCC8* mutations.[6,9,19,20]

Mutations identified in *KCNJ11* gene were concentrated in the N-terminal and the C-terminal regions of gene both of which form cytoplasmic ATP binding site of K_{ATP} channel. The residues R50, R201, and G334 are located in the main pockets of ATP binding sites and hence are functionally important.

In particular in our study the mutation R201 residue was identified in 11 of 20 patients pointing that this is one of the frequent mutations in Indian patients with PNDM. Interestingly, six patients had R201H mutation, five had R201C mutation and compared with R201H patients, the patients with R201C mutation had higher random blood sugar.

The severity of the disease phenotype is known to reflect the channel dysfunction. Previous study by Flanagan et al (2006) has shown a definite genotype- phenotype correlation. We observed a similar pattern in our study as well. All the *KCNJ11* mutations identified in this study were PNDM or DEND syndrome. A prominent feature among patients with *KCNJ11* mutations is that some of them manifest neurological dysfunction. In our series, three patients manifested DEND syndrome; two with V59M mutation and one with V64M mutation. The severity of developmental delay and epileptic seizures were greater in the patient with V64M compared with V59M. The DEND patient who harbored V64M did not respond to SU and continued to be on insulin and valproic acid for epilepsy, with EEG readings showing abnormality. In contrast, the two patients with V59M responded to SU and so were weaned off insulin and continue to be on SU. In the beginning they were administered valproic acid as treatment for epilepsy (Table 2). Subsequently they did not exhibit any episode of seizures, nor were the EEG readings abnormal and hence currently are being managed without valproic acid.

With regard to *ABCC8* gene, as shown in Figure 1, the 15 mutations are spread across the gene; however five mutations are concentrated in transmembrane domain 2 (TMD2). Of the four novel mutations two lie in the TMD2 region and two in nucleotide binding domain 2 (NBD2) region, both of which are functionally significant. The TMD2 region forms a critical site for potassium channel opener and is both physically and functionally important region. In our recent study,[21] mutations D212Y, R992C were functionally characterized using various experiments that confirmed them to be activating mutations resulting in increased channel function. Furthermore, mutation D212Y results in decreased channel sensitivity to ATP and biogenesis of K_{ATP} channel. Both these mutations respond to sulfonylurea drugs facilitating the transfer of the patients with these mutations in the present study. Pedigree analyses show that mutation H1537R co-segregates with diabetes in the family (IND-39) although the mother

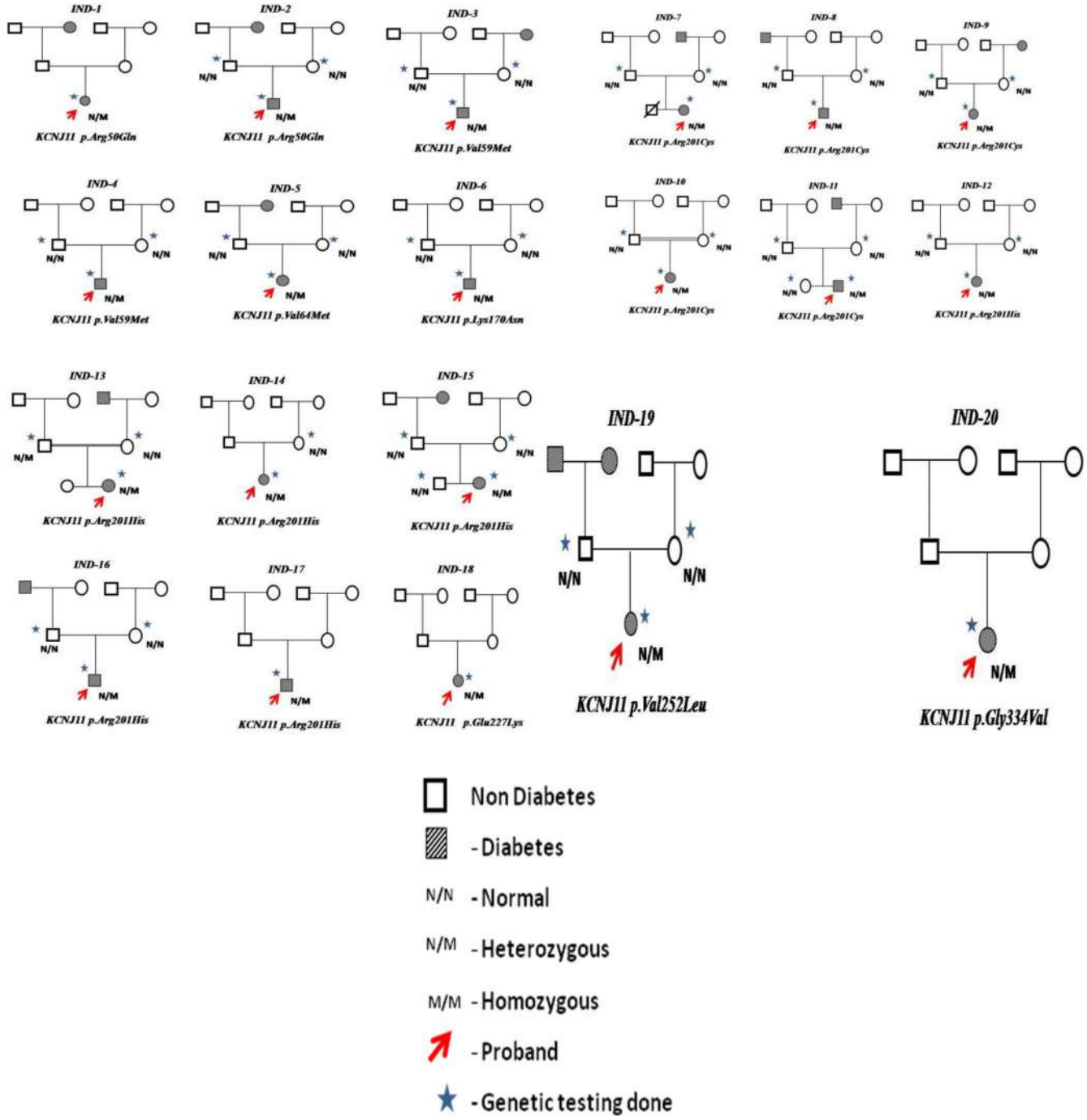

FIGURE 2 Pedigrees of neonates with *KCNJ11* gene mutations. Pedigrees of the families showing diabetes status of each member, as well as genetic status: M/M (homozygous mutant), M/N (heterozygous), and N/N (homozygous normal)

did not have NDM but had diabetes. Another novel mutation was frameshift stop gained mutation seen in homozygous condition in a patient with the parents being heterozygous carriers. In general this type of frameshift mutation results in premature termination of codons which is predicted to cause loss of function mutations resulting in recessive hypoglycemia. Surprisingly, we did not find this phenotype in the patient we have described in this study. We presume that the deletion mutation causing LOF may initiate alternate promoter to transcribe a non-coding RNA to form excess functional protein that can form a channel resulting in gain of function. However, only functional studies will help in elucidating the exact mechanism underlying the cause of this mutation. But whatever the mechanism, the favorable response to SU in this patient indicates that it is the K_{ATP} mutation and not some other mutation in another gene locus is responsible for this phenotype in the patient.

In patients with *ABCC8* mutations only two iDEND patients out of four could be shifted to SU. The success rate of transfer is more in *KCNJ11* compared with *ABCC8*. In this study we have sequenced only

TABLE 3 Clinical characteristics of patients with *ABCC8* gene mutation

Patients	*IND 21*	*IND 22*	*IND 23*	*IND 24*	*IND 25*	*IND 26*	*IND 27*	*IND 28*	*IND 29*	*IND 30*
Phenotype	PNDM	PNDM	PNDM	PNDM	PNDM	PNDM	PNDM	PNDM	PNDM	PNDM
Gender	F	F	M	M	M	M	F	F	M	M
Current age (years)	5	3.3	8.2	2.10	5.5	7.7	5.2	5.6	4.10	6.3
Age at diagnosis (months)	3.5	4.5	3	3	1.5	4	5	3	2	2.5
Birth weight (g)	2450	2300	2500	2750	2500	2500	2750	3000	2500	2500
Birth weight (centiles)	_	_	50	75	50	_	_	99	50	50
RBS (mg/dL)	411	450	207	164	396	246	404	600	399	487
Consanguinity	Yes	No	No	No	No	No	No	No	No	No
Developmental delay	Yes	Yes	Yes	Yes	No	No	No	No	No	No
Mutations										
cDNA	c.145A>T	c.394T>C	c.394T>C	c.634G>T	c.643G>A	c.643G>A	c.674T>C	c.674T>C	c.1608T>A	c.2974C>T
Zygosity	Hetero	Hetero	Hetero	Hetero	Hetero	Hetero	Hetero	Hetero	Hetero	Hetero
Protein	p.I49F	p.F132L	p.F132L	p.D212Y	p.V215I	p.V215I	p.L225P	p.L225P	p.F536L	p.R992C
Novel	No	No	No	No	No	No	No	No	No	No
DKA at presentation	—	—	No	No	No	—	—	Yes	Yes	No
Insulin therapy after clinical diagnosis (U/kg/day)	3	4	4	5	5	14	8	8	9	5
SU treatment	+	_	_	+	+	+	+	+	_	+
SU treatment initiated (years)	1	_	_	0.4	0.8	6.6	1	0.6	_	0.5
Response	Yes	No	No	Yes	Yes	Yes	Yes	Yes	No	Yes
Other medication	No	Insulin	Insulin	Valproic acid	No	No	No	No	Insulin	No
RBS after shifting (mg/dL)	126	143	135	157	124	146	125	125	154	120
RBS during recent follow up (mg/dL)	115	138	131	138	100	117	100	113	141	109
Follow up (years)	4	_	_	2.6	4.7	1.1	4.2	5	–	5.8

TABLE 3 Clinical characteristics of patients with *ABCC8* gene mutation (Contd…)

Patients	*IND 31*	*IND 32*	*IND 33*	*IND 34*	*IND 35*	*IND 36*	*IND 37*	*IND 38*	*IND 39*
Phenotype	PNDM	PNDM	PNDM	PNDM	PNDM	PNDM	PNDM	PNDM	PNDM
Gender	M	F	M	M	M	F	F	F	M
Current age (years)	5.7	2.10	4.10	7.5	3.10	0.5	5.4	5	4.10
Age at diagnosis (months)	3.5	5	1	3	1.5	1	1	6	4
Birth weight(g)	2250	3000	1160	3000	2800	2800	2480	2200	1900
Birth weight (centiles)	—	99	99	—	75	90	75	—	3
RBS (mg/dL)	264	402	500	750	560	410	457	511	400
Consanguinity	No	No	No	No	Yes	Yes	No	Yes	No
Developmental delay	Yes	No	No	No	No	Yes	No	Yes	No
Mutations									
cDNA	c.3544C>T	c.3544C>T	c.3544C>T	c.3593C>T	c.3788C>T	c.3381 del T	c.3860A>G	c.4264T>A	c.208G>Cc.4610A>G
Zygosity	Hetero	Hetero	Hetero	Hetero	Homo	Homo	Hetero	Hetero	Compound hetero
Protein	p.R1182W	p.R1182W	p.R1182W	p.P1198L	p.A1263V	p.D1128Tfs*16	p.Y1287C	p.S1422T	p.G70R, p.H1537R
Novel	No	No	No	No	No	Yes	Yes	Yes	No, Yes
DKA at presentation	_	Yes	No	_	No	Yes	Yes	_	No
Insulin therapy after clinical diagnosis (U/kg/day)	8	18	5	14	20	3	4	2	6
SU treatment	+	+	+	+	+	+	_	_	+
SU treatment initiated (years)	0.10	1.1	0.3	6.2	0.10	0.3	_	_	3.7
Response	Yes	Yes	Yes	Yes	Yes	Yes	No	No	Yes
Other medication	No	No	No	No	No	No	Insulin	Insulin	No
RBS after shifting (mg/dL)	137	143	138	132	127	125	123	135	132
RBS during recent follow up (mg/dL)	121	126	131	121	116	108	105	116	120
Follow up (years)	3.9	1.9	4.7	1.3	3	0.2	—	—	1.3

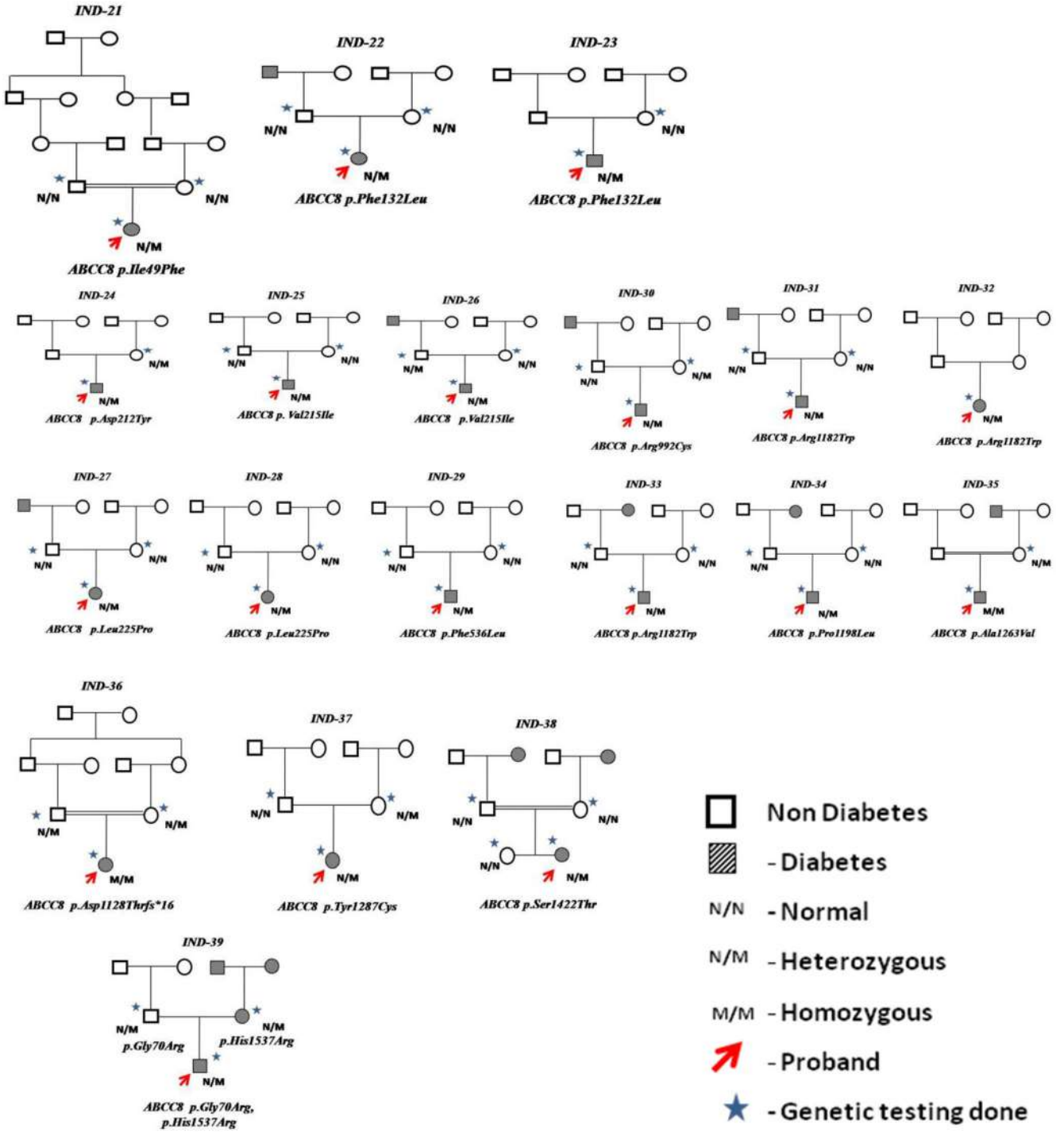

FIGURE 3 Pedigrees of neonates with *ABCC8* gene mutations. Pedigrees of the families showing diabetes status of each member, as well as genetic status: M/M (homozygous mutant), M/N (heterozygous), and N/N (homozygous normal)

the K_{ATP} channel genes and not the non-K_{ATP} channel genes which also cause NDM, albeit in lesser frequency. This is a limitation of this study.

From clinical perspective it is very important to identify mutations in *KCNJ11* and *ABCC8* genes in patients with PNDM since some of these mutations are responsive to sulfonylurea therapy, which improves the diabetic control dramatically. In our study, 33 of 39 patients (84%) responded to SU treatment and shifting of patients from insulin completely to SU was possible for them. The shifting protocol used was from an earlier study by Pearson et al.[22]

This study is significant in many ways. This is the first large study describing the spectrum of mutations in K_{ATP} channel NDM in India. It has demonstrated the importance of K_{ATP} channel gene screening in PNDM and efficacy of glibenclamide for Indian patients with K_{ATP}-PNDM. This study has delivered on precision diabetes to 84% of the

Indian PNDM patients. Further, the reported rate of K_{ATP} channel mutations is much lesser in proportion in Indians compared with Caucasian and Japanese populations. The unknown genetic etiology in about 78% of PNDM patients underscores the importance of investigating non-K_{ATP} channel genes as well as the immediate need to search for novel genes and genetic etiology.

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