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ORIGINAL ARTICLE

Maturity onset diabetes of the young in India – a distinctive mutation pattern identified through targeted next-generation sequencing

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Summary

Objective To establish and utilize a Next-Generation Sequencing (NGS)-based strategy to screen for maturity onset diabetes of the young (MODY) gene mutations in subjects with early-onset diabetes.

Patients and Methods Maturity onset diabetes of the young (MODY) genetic testing was carried out in 80 subjects of Asian Indian origin with young onset diabetes to identify mutations in a comprehensive panel of ten MODY genes. A novel multiplex polymerase chain reaction (PCR)-based target enrichment was established, followed by NGS on the Ion Torrent Personal Genome Machine (PGM). All the mutations and rare variants were confirmed by Sanger sequencing.

Results We identified mutations in 11 (19%) of the 56 clinically diagnosed MODY subjects and seven of these mutations were novel. The identified mutations include p.H241Q, p.E59Q, c.-162G>A 5′ UTR in *NEUROD1*, p.V169I cosegregating with c.493-4G>A and c.493-20C>T, p.E271K in *HNF4A*, p.A501S in *HNF1A*, p.E440X in *GCK*, p.V177M in *PDX1*, p.L92F in *HNF1B* and p.R31L in *PAX4* genes. Interestingly, two patients with *NEUROD1* mutation were also positive for the p.E224K mutation in *PDX1* gene. These patients with coexisting *NEUROD1–PDX1* mutations showed a marked reduction in glucose-induced insulin secretion. All 24 subjects who had not met the clinical criteria of MODY were negative for the mutations. To the best of our knowledge, this is the first report of *PDX1*, *HNF1B*, *NEUROD1* and *PAX4* mutations from India.

Conclusions Multiplex PCR coupled with NGS provides a rapid, cost-effective and accurate method for comprehensive parallelized genetic testing of MODY. When compared to earlier reports, we have identified a higher frequency and a novel digenic mutation pattern involving *NEUROD1* and *PDX1* genes.

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Introduction

Maturity onset diabetes of the young (MODY) is an autosomal dominant monogenic disorder characterised by beta-cell dysfunction.[1] MODY accounts for up to 2% of patients with diabetes[2] and in India, with the current estimated prevalence of 62·4 million with diabetes and 77·2 million with prediabetes[3] there could be considerable number of patients with MODY. Though MODY has been traditionally perceived to present before the age of 25 years,[4] the diagnosis of diabetes can be delayed beyond 35 years.[2] There has also been a trend towards a shift in the mean age of onset of type 2 diabetes (T2D) to a much younger age, that ranges between 25 and 34 years.[3] The overlapping clinical features of MODY with the classical polygenic diabetes present a diagnostic challenge and require genetic testing for differentiation of MODY.[5]

MODY mutations in around twelve different genes have been reported to date. The current consensus relating to genetic diagnosis relies on phenotype-guided screening of the most commonly implicated genes in MODY. These include hepatocyte nuclear factor 1alpha (*HNF1A*), glucokinase (*GCK*) and hepatocyte nuclear factor 4 alpha (*HNF4A*) genes.[2,6] Subjects negative for mutations in these genes are considered for screening of genes implicated in other rarer forms of MODY.[2] However, due to limitations with access to genetic diagnostic facilities, high cost and a relative lack of clinician awareness,[7] this subset of patients with diabetes are often misdiagnosed as either T1D or T2D[8] and may potentially receive inappropriate therapy.[9]

Furthermore, the clinical phenotype in individuals with the same form of MODY and also members within a family who

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share the same mutation may vary significantly.[4] However, due to the limitations associated with the potential scalability of Sanger sequencing, multigene testing able to provide a more comprehensive genetic portrait in a heterogenous disorder like MODY has not been feasible in the past. With the advent of high-throughput next-generation sequencing (NGS) technology, there has been a significant improvement in sequencing strategies enabling simultaneous analysis of a panel of genes.[10] Therefore, utilizing the NGS approach, the current study was aimed at screening ten MODY-related genes in a group of subjects with young onset diabetes.

Methods

Study population

Based on previous studies,[4,8] a 20% cumulative mutation rate was assumed, and with a precision of ±8%, we recruited 80 subjects with young onset diabetes (age of diagnosis before 35 years), of whom 56 (group I) were clinically diagnosed as MODY. Clinical diagnosis of MODY was considered when a subject with young onset diabetes exhibited an autosomal dominant pattern of inheritance, absence of β-cell autoimmunity and diabetic ketoacidosis (DKA).[5,8] In the remaining 24 (Group II) subjects who did not meet the clinical criteria of MODY, eleven were without autosomal dominant pattern of inheritance, nine with features of insulin resistance and four with DKA at onset (Fig. 1). Further, sixty subjects with normal glucose tolerance were selected as controls for the validation of the novel pathogenic variants. Subjects were recruited from the outpatient endocrinology clinics of Christian Medical College and Hospital, Vellore between March 2012 and October 2013.

This study was approved by the Institutional Review Board (IRB) and Ethics Committee of Christian Medical College, Vellore (IRB Min. No. 7774 dated 22nd February, 2012). Written informed consent was obtained from all study participants and healthy control subjects.

Sequencing capture and DNA sequencing (2GDMODY Workflow)

Target enrichment. We developed a novel multiplex PCR-based enrichment for ten MODY genes using a QIAGEN® (Hilden, Germany) Multiplex PCR kit, followed by sequencing on Ion Torrent™ Personal Genome Machine (PGM) (Fig. 2). The panel of genes included *HNF1A, HNF4A, GCK*, pancreatic and duodenal homeobox 1 (*PDX1*), hepatocyte nuclear factor 1beta (*HNF1B*), neurogenic differentiation factor 1 (*NEUROD1*), kruppel-like factor 11 (*KLF11*), carboxyl ester lipase (*CEL*), paired box 4 (*PAX4*) and insulin (*INS*). The Primer 3 software was used to design 57 primer sets covering the 34·4 kb target of these ten genes (except pseudo gene regions of *CEL*). The primers included an additional 50 bp both upstream and downstream of each exon to capture the splice junctions and also the 5′ UTR and 3′UTR regions. The primer sequences used in this study are given in Table 1. The cycling conditions were: 10 min at 95 °C, 5 min at 98 °C, 25 cycles of 30 s at 98°C, 90 s at 60 °C, 90 s at 72 °C and final elongation 10 min at 72 °C.

Library preparation. Enzymatic shearing of pooled amplicons, adaptor ligation, nick repair and amplification was performed using the Ion Xpress™ Plus Fragment Library Kit (Life Technologies, Grand Island, NY, USA). The sheared DNA was further ligated with common adaptors for single samples and Ion Xpress™ barcode adapters (Ion Torrent, Life Technologies) for multiplex samples. The ligated samples were size-selected on E-Gel® Size Select™ 2% precast agarose gels. These libraries were further amplified for 4–6 cycles of PCR. The Agilent bioanalyzer high sensitivity DNA chip (Agilent Technologies, Santa Clara, CA, USA) was used to determine the quality and concentration of the libraries. The concentration was further confirmed by a TaqMan® Quantitation Kit (Ion Torrent, Life Technologies).

Template preparation. The clonal amplification (template preparation) was performed on Ion OneTouch Emulsion PCR using the Ion OneTouch™ 200 Template Kit/Template Kit v2 DL (Ion Torrent, Life Technologies). This was followed by a streptavidin-coupled Dynabeads®-based enrichment on an Ion OneTouch Enrichment System (Ion Torrent, Life Technologies) prior to sequencing.

Sequencing and bioinformatic analysis. Sequencing was performed on Ion Torrent PGM using Ion PGM™ 200 Sequencing Kit (Ion Torrent, Life Technologies), utilizing 314 chips (multiplex 3 samples) and 316 chips (multiplex 8–10

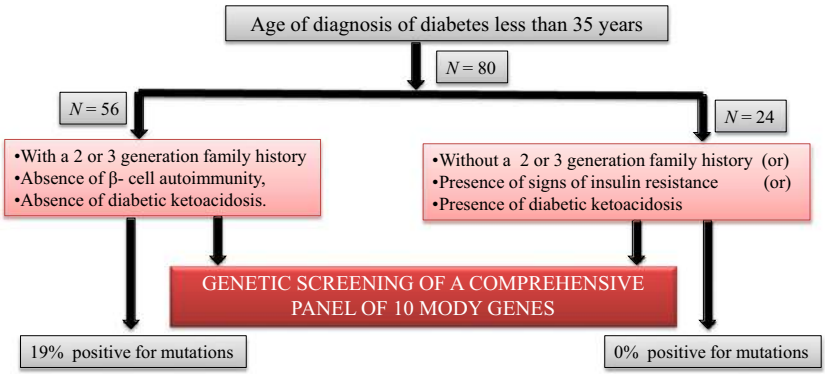

Fig. 1 Algorithm classifying the subjects with young onset diabetes.

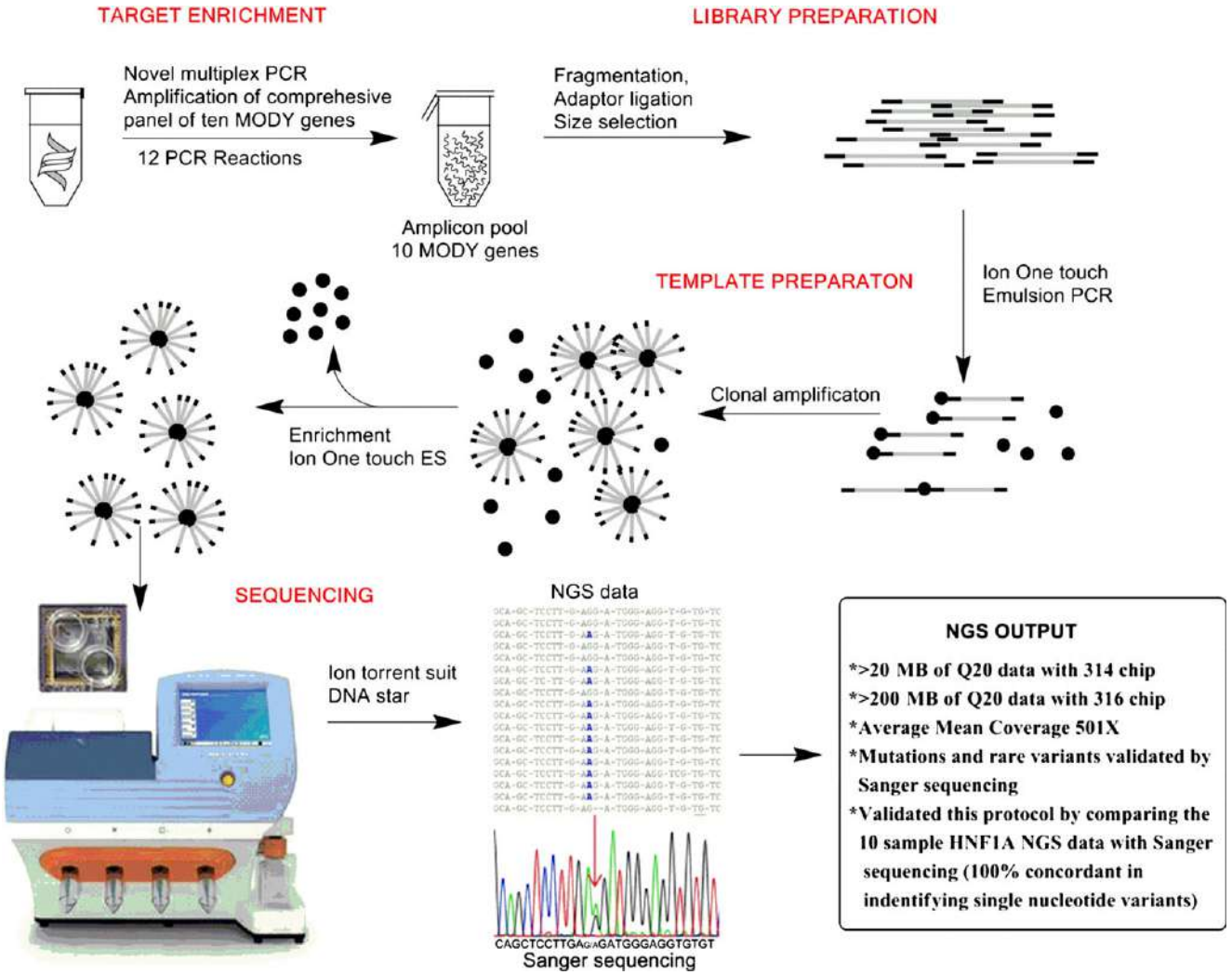

Fig. 2 Second generation genetic diagnosis of MODY (2GDMODY) work flow. (1) Target Enrichment: Novel multiplex PCR approach to amplify ten genes in 12 PCR reactions. (2) Library Preparation: Ion Xpress™ Plus Fragment Library Kit was utilized to shear the amplicon pool, adaptor ligation, size selection with 2% egel size select followed by amplification. Agilent 2100 DNA High sensitivity chip and TaqMan assay were utilized for quantification of the libraries. (3) Template preparation: Template preparation was performed on Ion One Touch Emulsion PCR system, followed by enrichment on Ion One Touch Enrichment System. (4) Next-generation sequencing: Performed on Ion Torrent PGM with 314, 316 Ion chips and 200 bp sequencing kits. Ion torrent suit software and DNA star software were utilized for data analysis. Sanger sequencing was performed to validate the protocol and the results.

samples). The generated sequencing data were mapped to the human genome reference hg19 using Torrent Mapping Alignment Program (TMAP). The Torrent suit software with v2.0 and/or v3.6.2 (Life Technologies) was used for all analysis. The coverage analysis was calculated using Torrent Coverage Analysis, and potential pathogenic variants were identified using the Torrent Variant Caller (Figure S1). In addition, we utilized DNA STAR software (DNASTAR, Madison, WI, USA) for further data analysis.

Validation. Sanger sequencing was performed to confirm all identified mutations and rare variants. These novel mutations were absent in sixty control subjects. All novel variants were evaluated for sequence conservation, and the likelihood of pathogenicity using PolyPhen-2, Sorting Intolerant From Tolerant (SIFT), Mutation taster and Genomic Evolutionary Rate Profiling (GERP) and The Human Gene Mutation Database (HGMD® Professional 2012.4) was utilized to classify identified variants as reported or novel.

Results

In this study, a comprehensive panel of ten MODY genes were screened in 80 patients with young onset diabetes: 42 men and 38 women. The mean age of these subjects at the time of diagnosis of diabetes was 24·1 ± 6·7 years with 38 of these subjects being diagnosed between the ages of 26–35 years. Among the MODY mutation-positive subjects, the

Table 1. 2GDMODY primer sequences

Oligo	Bases	Sequences	Oligo	Bases	Sequences
HNF4A_1F	20	CACAGTTCTCCACCCTCCTT	IPF1-1R	20	TTAGTCCGACCCGGGATAAT
HNF4A_1R	20	CTTAGGGAAGCGGTCACATT	IPF1-2F	20	GGAAGGGCTTGAGTTACTAGGG
HNF4A_2F	20	AGGTGATGGAGTGGGAACAG	IPF1-2R	20	GGTTTTCCCCTTCGGTCTAA
HNF4A_2R	21	CAAAACCAGAAGAGCCTTGAA	HNF1B_1F	21	GCACTGGCTTAACAAGTCCAA
HNF4A_3F	20	AACTCCCGGGATGAAGAGAT	HNF1B_1R	20	GACTTCTCTGGTGGGAAACG
HNF4A_3R	20	TCTCAGCCATTAGCCAGTCA	HNF1B_2F	20	GTACAGGGGCAGTCACCTT
HNF4A_4F	20	CTGATGTGGGCCTGTTCTC	HNF1B_2R	20	ATCTGCCAAGTGCTCACAAG
HNF4A_4R	20	GGAGAATGGAGGTGGAGGA	HNF1B_3F	20	AAAGGTGTCTTCGTCCGTTG
HNF4A_5F	20	CTCCCTCCCTCCGTTTTTAC	HNF1B_3R	20	TGGGGTTCTGTGGAACATACT
HNF4A_5R	23	CCACGGCTATATCCCAGGT	HNF1B_4F	20	CCCTTCATACTCCCAACCAA
HNF4A_6F	20	CAGTTCAGGCAGGTAGAGGC	HNF1B_4R	20	GCGTTTGCTCCTCTGAAAAC
HNF4A_6R	20	GTGGGGTACCCAGTTGAAAA	HNF1B_5F	20	AGAGGTGCCGAGTCATTGTT
HNF4A_7F	20	CCTGTGAAATGGGAGTCACC	HNF1B_5R	20	CAGCCCTCATTTTCCCCTAT
HNF4A_7R	20	AAATGAAAACGGCCTCTCCT	HNF1B_6F	20	GTGAATGAAGGAATGAATTTGAG
HNF4A_8F	20	ACAAGTCAGGGGACATCTG	HNF1B_6R	20	GTCGTGGGTGAGTTTGAAGG
HNF4A_8R	20	ACGTCCTCCATCTCACAACC	HNF1B_7F	19	GCATCCATCCACCTCTCCT
HNF4A_9F	20	TATTGGATGGGCTGGTTGAT	HNF1B_7R	20	TTCAGACCCAGAGAGGGAAA
HNF4A_9R	20	ACCCTGGAACCCAGAAAACT	HNF1B_8F	20	TGAGGAGATGGGAGCTATGG
HNF4A_10F	23	GATGGAGGAGATGGGTGGTA	HNF1B_8R	20	AACAACAGGGAGCCTCAGAA
HNF4A_10R	20	CAGGGATCCTCACCCAAGTA	HNF1B_9F	20	GAAGTGTGCCTCAGCATGAA
GCK_1F	20	TGCATGGCAGCTCTAATGAC	HNF1B_9R	20	CAGAAAATTCCAAGGAGACCA
GCK_1R	20	CTGGCAAGACCCTTCTCAAA	NEUROD1-1F	20	CTCTATCCCCGTCCCTTCTG
GCK_2F	18	GTGTGCAGATGCCTGGTG	NEUROD1-1R	20	GGATGCTGGTCTCAATACACA
GCK_2R	20	CTGGCTGTGAGTCTGGGAGT	NEUROD12AF	20	GAAGCTGAAGGCGTATCTGG
GCK_3F	20	CCTTCCCTCCTCCTCTTTGT	NEUROD12AR	20	GTGGAAGACATGGGAGCTGT
GCK_3R	19	ACCTCCCGTCAGGACTAGC	NEUROD12BF	20	CTCGGACTTTTCTGCCTGAG
GCK_4F	20	TTGCAGTGTCCCTGAGGAAT	NEUROD12BR	21	CGATCTGAATACAGCCACACC
GCK_4R	20	GGGCTACATTTGAAGGCAGA	KLF11-1F	18	GCGCGGTGTATTTTGGTT
GCK_5F	20	CCCTGTGCAGGAGGTAGTGA	KLF11-1R	18	GAAGCGCAAGACAAAGCTG
GCK_5R	20	TCAAAGTCCTGCCAAGAAGC	KLF11-2F	22	TTGTCATGAGCATTCCTTGAA
GCK_6F	20	TATCAAACGGAGAGGGGTGA	KLF11-2R	20	CTCACAAGTTCTTCAACACACTGA
GCK_6R	19	GCCCTTGAAGCCTGTTGTA	KLF11-3F	23	TGTAAAGGTATTGGGAGCATTGT
GCK_7F	19	GACTCCTGTGGGCAGGAAC	KLF11-3R	19	CCAGGGGTTCTCATGATCC
GCK_7R	20	TTTGCTTTTCCCCAGAGTTG	KLF11-4F	23	TCTTCTGAGTTTGATGAACACGA
GCK_8F	19	GAGGGAAAGACGTGAACCAG	KLF11-4R	23	AGGCCAGATAACTTGTGGTTTTT
GCK_8R	20	CTGAGACCAAGTCTGCAGTG	CEL-1F	19	AAGCAGGGCATGGAGACAT
GCK_9F	18	AGGAGGCTGGGACAGGAC	CEL-1R	20	GAATTGGGCATCCTGTGTTC
GCK_9R	20	AATCTTGGAGCTTGGGAACC	CEL-234F	20	GAAGCTGTCTTTGCCTCTGG
GCK_10F	20	CTAGATTTGGGGGAAGGGTC	CEL-234R	20	CTACTCCAGCTTCCGGGTCT
GCK_10R	20	CGCTATGGGAGCTGAAGATG	CEL-567F	20	CCAACTCTGTTGAGGGCATT
HNF1A_1F	19	CAGGCCATAGCTCCCTGTC	CEL-567R	20	CTGGCACCTTCCTCTCTCCT
HNF1A_1R	20	TCAAACCTCCAAGCAAGGAC	PAX4-12F	20	GTTCTGGGGAAGCTTTTTCC
HNF1A_2F	20	GGGCTCCATAACTGCTTTCA	PAX4-12R	21	CCCACATTGCCAAATAAGAAA
HNF1A_2R	20	GCAGGTTGAATCCCACTGAC	PAX4-3F	20	GGTGGAGACAGATGGGAAAA
HNF1A_3F	21	TGTGCTGTGTGTTTGTCATCA	PAX4-3R	20	TTGTTGTGAGGGTGATCCAA
HNF1A_3R	20	GCCAGGCTAAGCCAATATCA	PAX4-456F	20	CAGCTTGGCTCTGCTCTTCT
HNF1A_4F	20	GACTGTCAATTGCCCAAGGT	PAX4-456R	19	AAATGGATGGACGAATGGAG
HNF1A_4R	20	AAGGAGTGGCATGAATGGAA	PAX4-78F	20	ATCCAACCTTCCTGCCTCTC
HNF1A_5F	20	CTGGCCTAAGCAAACCAATG	PAX4-78R	20	TGAATATTAGAGTGGGCATAGGG
HNF1A_5R	20	GAGGGGAAGCCAAGCTGT	PAX4-9F	20	ATATGCAGGGTGGGAAACTG
HNF1A_6F	20	TGCTGAGTACAGAAGCCAAGC	PAX4-9R	23	CTTCTCAGGAAGGGCCTAGA
HNF1A_6R	20	ACCACCTCTCCTTCCCAGAG	INS-12F	20	CTGTGAGCAGGGACAGGTCT
HNF1A_7F	20	AAAGAGCTAAAGGCTCAGAGAGG	INS-12R	22	CACTTTTAGGACGTGACCAAGA
HNF1A_7R	20	GTCCCAGAGACACATGCAGA	INS-3F	20	CCCTGACTGTGTCCTCCTGT
HNF1A_8&9F	20	CCAGTTTTGAAAATCAGCCC	INS-3R	20	AGAGAGCGTGGAGAGAGCTG
HNF1A_8&9R	19	AGTGCTTCCTCACAGCAGC			
HNF1A_10F	20	TGAGTACCCCTAGGGACAGG			
HNF1A_10R	18	CAGGCTCAGGTCCTCCAG			
IPF1-1F	20	CCTGGGCCTAGCCTCTTAGT			

mean age at diagnosis was 22 ± 7·7 years with five subjects diagnosed between the age of 26–30 years. The average BMI of the patients (n = 77) was 24·6 ± 4·8 kg/m^2. According to Asian Indian obesity cut-offs,[11] 28 subjects were normal weight, 18 subjects were overweight and 25 subjects were obese. Six subjects had a BMI of less than 18·5 kg/m^2. The mean BMI in subjects who carried a MODY mutation was 25 ± 5·8 kg/m^2 and five subjects were normal weight, one subject was overweight and five subjects were obese. Only one of the eleven subjects who had a BMI of more than 30 kg/m^2 was positive for a MODY mutation. Among the 23 individuals who were on insulin therapy, five subjects were positive for MODY mutations. Using NGS, we identified mutations in 19% (11/56 in group I) of the clinically diagnosed MODY subjects, seven of which were novel (Fig. 3). There were no MODY positive mutations in Group II, which consisted of 24 subjects who did not fit into the clinical criteria of MODY. There were two mutations in *NEUROD1*, two in *HNF4A*, one each in *GCK, HNF1A, PDX1, HNF1B* and *PAX4* genes. Furthermore, in two patients, a novel digenic *NEUROD1–PDX1* mutation pattern was detected (Fig. 3).

When compared to previous studies,[12,13] a higher frequency of *NEUROD1* mutations (36%) was identified among the mutation-positive subjects in our study population. In addition to the previously reported H241Q *NEUROD1*mutation[13] in subjects M26 and M47, two novel *NEUROD1* mutations identified include 5′UTR c.-162G>A and E59Q in subjects M30 and M18, respectively. Interestingly two patients M30 and M26 with *NEUROD1* mutation were also positive for the p.E224K mutation in *PDX1* gene.[14]

Subject M30 with the digenic 5′UTR c.-162G>A *NEUROD1* and E224K *PDX1* mutation, had an autosomal dominant pattern of inheritance from his maternal and paternal side (Fig. 4a: M30-NP2 pedigree). M30 inherited the novel 5′UTR c.-162G>A *NEUROD1* mutation from his father and the E224K *PDX1* mutation from his mother who were diagnosed with diabetes at the age of 51 and 35 years, respectively. Among the family members the age of onset of diabetes varied from 35 to 55 years. The patient's paternal relatives were obese with a BMI more than 30 kg/m^2. They were diagnosed with diabetes after the age of 40 years and are responding to SU therapy, whereas his maternal relatives with a normal BMI had earlier age of onset (<40 years)

UMEPN	Sex	Age	AOD	BMI	TREATMENT	GENE	MODY	Mutation	Aminoacid change	GERP	SIFT	PP	M T	COMMENT
Reported Mutations														
M10	M	23	21	25	Metformin & Sitagliptin	HNF4A	MODY1	c.505G>A c.493-4G>A (splice site mutation) c.493-20C>T	p.Val169Ile					Gragnoli et al., (2004)
M15	F	8	8		Diet	GCK	MODY2	c.1318G>T	p.Glu440X					Osbak et al., (2009)
M26	M	47	28	22·8	Glimepiride	NEUROD1	MODY	c.723C>G	p.His241Gln					Gonsorcikova et al., (2008)
						PDX1		c.670G>A rs137852787	p.Glu224Lys					Cockburn et al., (2004)
M47	F	35	24	39·7	Metformin & Glimpride	NEUROD1	MODY6	c.723C>G	p.His241Gln					Gonsorcikova et al., (2008)
Novel Pathogenic Variants/ Mutations														
M85	F	37	27	21·7	Insulin	HNF4A	MODY1	c.811G>A	p.Glu271Lys	4·98	D	PD	DC	Likely pathogenic
M01	F	21	11	19·1	Insulin & Glibenclamide	HNF1A	MODY3	c.1501G>T	p.Ala501Ser	4·49	T	PD	DC	Likely pathogenic
M62	M	40	26	24·4	Insulin	PDX1	MODY4	c.529G>A	p.Val177Met	4·86	D	PD	DC	Likely pathogenic
M19	F	27	25	26·3	Metformin & Insulin	HNF1B	MODY5	c.274C>T	p.Leu92Phe	5·13	T	PD	DC	Likely pathogenic
M18	M	30	30	19·31	Glimepiride	NEUROD1	MODY6	c.175 G>C	p.Glu59Gln	5·9	T	BN	DC	Likely pathogenic
M30	M	30	30	27·5	Metformin & Glipizide	NEUROD1	MODY	c.-162G>A 5'UTR		4·05			DC	Likely pathogenic
						PDX1		c.670G>A rs137852787	p.Glu224Lys	4·01	D	PD	DC	Cockburn et al., (2004)
M33	M	14	14	23	Glimepiride & Insulin	PAX4	MODY9	c.92G>T	p.Arg31Leu	5·73	D	PD	DC	Likely pathogenic

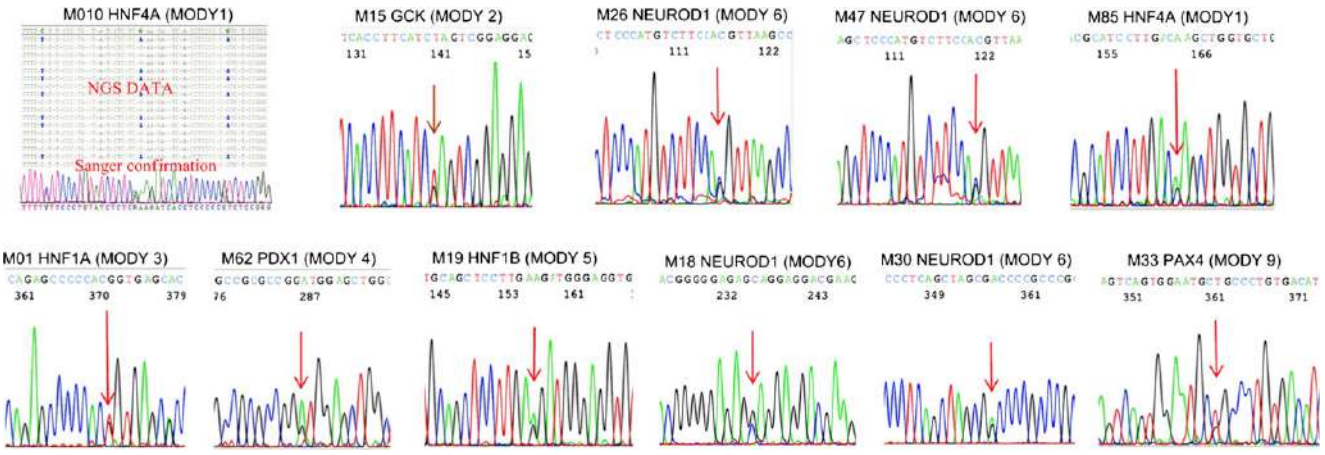

Fig. 3 Mutations identified by targeted next-generation sequencing and Sanger Confirmation. UMEPN, Unique molecular endocrinology pin number; AOD, Age of diagnosis; GERP, Genomic evolutionary rate profile; D, Damaging; T, Tolerated; PD, Probably damaging; BMI, Body mass index; PP, POLYPHEN; MT, Mutation Taster; D, Damaging; PD, Probably damaging; DC, Disease causing; HNF4A, Hepatocyte Nuclear factor 4 alpha; GCK, Glucokinase; HNF1A, Hepatocyte nuclear factor 1 alpha; PDX1 or IPF1, Insulin promoter factor 1; HNF1B, Hepatocyte nuclear factor 1 beta; NEUROD1, Neurogenic differentiation factor 1; PAX4: Paired box 4.

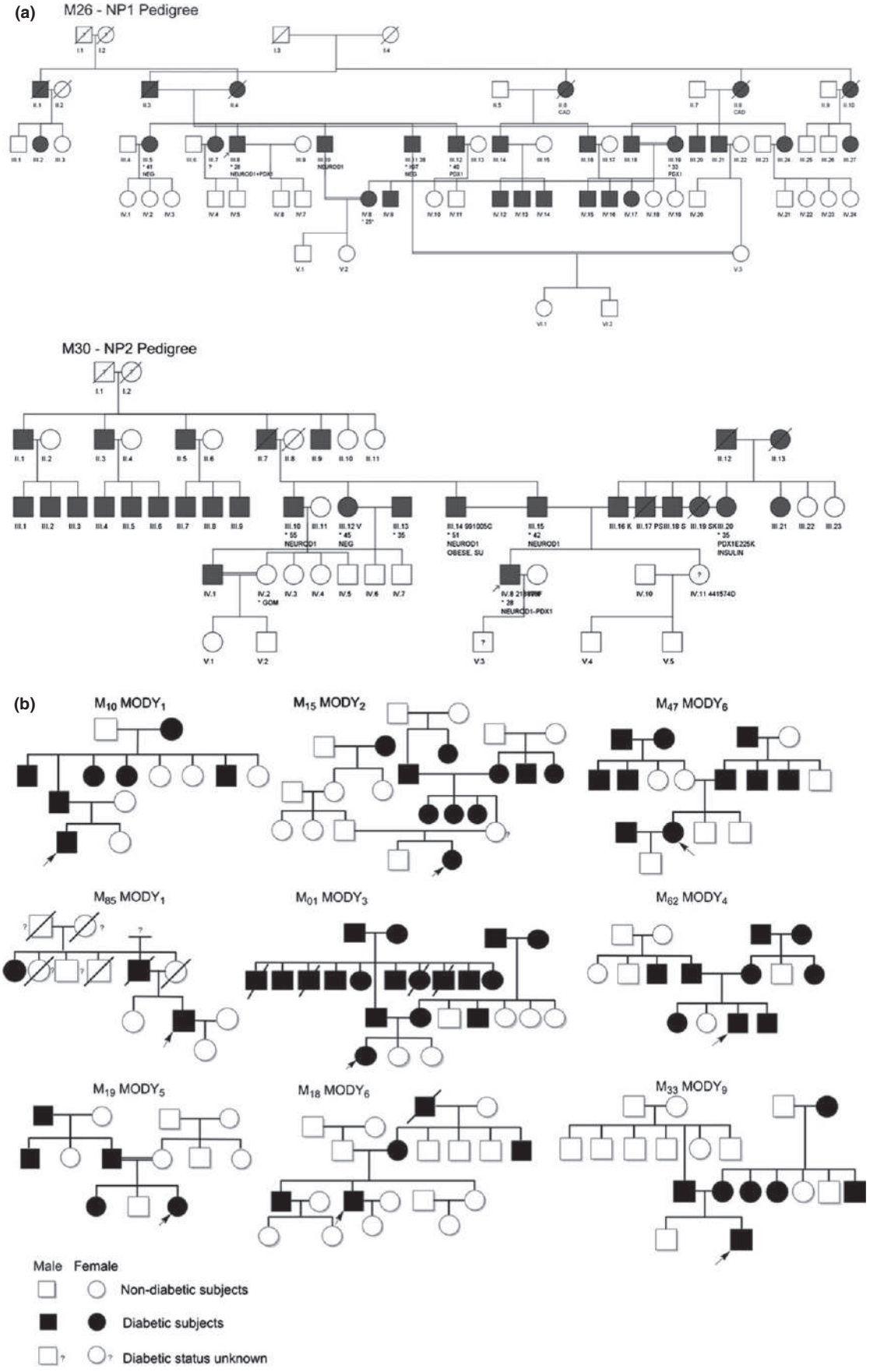

Fig. 4 (a) The pedigree of M26 and M30. *M26-NP1 Pedigree*: Proband M28 (III.8) is a 47-year-old subject diagnosed to have diabetes at the age of 28. With a coexisting *NEUROD1–PDX1* mutation he was on insulin for ~10 years and currently being managed with SU therapy. Both his parents (expired) and all his father's siblings are diabetic with variable phenotypes. Mutation screening of 5 of his siblings was performed for the digenic mutation. Two (III.12 & III.19) were positive for E224K *PDX-1* mutation and one (III.10) was positive for *NEUROD1* mutation and two were negative for either of the mutations. Four members have consanguineous marriage in the pedigree (only 3 shown). Samples for genetic analysis of rest of the family were unavailable at the time of submitting the article. All have responded to SU for at least 5 years except IV.8 who required Insulin since onset of diabetes. *M30- NP2 Pedigree*: Proband M30 (IV.8) with the coexisting *NEUROD1–PDX1* mutation was diagnosed with diabetes at the age of 28 years. He is now responding to SU therapy. Both his parents and most of the parents' siblings have diabetes with variable phenotypes. Mutation analysis was performed in parents. Father (III.14) is positive for Neurod1 5′ UTR mutation and mother (III.20) is positive for E224K *PDX-1* mutation. Further, the *NEUROD1* mutation was identified in two (III.10 & III15) of the three paternal siblings who consented for genetic testing. His maternal siblings are yet to be screened for *PDX1* mutation. All father's siblings were obese and have responded to SU therapy, and mother siblings have normal BMI but are requiring insulin for glycemic control within 5 years since onset of diabetes. (b) The pedigree charts of mutation-positive subjects.

and needed insulin within 5–10 years of diagnosis. Furthermore, the *NEUROD1* 5′ UTR mutation was identified in two of the three paternal siblings who consented for genetic testing. Maternal relatives are yet to give consent for genetic testing.

Additionally, subject M26 has a digenic H241Q *NEUROD1*–E224K *PDX1* mutation. His pedigree (Fig. 4a: M26-NP1 pedigree) revealed a strong autosomal dominant inheritance from the paternal side and had family members with a variable onset of diabetes from 38 to 50 years of age. Among the five siblings who were screened, two were positive for the *PDX1* mutation and one was positive for the *NEUROD1* mutation.

In M18, we identified a novel E59Q *NEUROD1* mutation in a highly conserved region with a GERP score of 5·9. With a three-generation family history, the proband, his sibling and his maternal uncle were diagnosed to have diabetes at the age of 30, 27 and 29 years, respectively. None of the family members were obese.

Mutations involving the *HNF4A* gene include a previously reported triple genetic variant with a missense mutation in exon 5 V169I of *HNF4A* cosegregating with intronic variants, IVS4-nt4 and IVS4-nt20.[15] This mutation is located in the ligand binding/dimerization domain of *HNF4A,* and IVS4-nt4 has been predicted to affect the splicing pattern.[15] There has been only one previous report of this triple genetic mutation in a Philippino family with early-onset T2D.[15] Furthermore, the V169I mutation was absent in 1270 patients with T2D and 1017 healthy nondiabetic European American individuals, and only one of the patients with T2D was positive for IVS4-nt4.[16] Other mutations involving *HNF4A* include a novel E271K mutation in M85, a male subject diagnosed to have diabetes at the age of 27 years.

M15 is an 8-year-old girl, who was diagnosed to have fasting hyperglycaemia following the evaluation for a urinary tract infection. With a confirmed *GCK* nonsense mutation E440X,[17] she was managed with dietary modification and exercise.[1,18] M01 is a 21-year-old female patient who had been on insulin therapy since her diagnosis with diabetes at the age of 11 years. We have identified a novel A501S mutation in the *HNF1A* gene, and at this codon, there exists a reported A501T mutation proven to be causative.[19] Addition of a low dose of sulphonylurea (SU) resulted in reduction of her insulin requirements. Furthermore, in contrast to earlier reports from other ethnic groups,[6] the prevalence of *HNF1A* mutations was only 9% in India[20] similarly, in the present study, we have identified only one subject with a *HNF1A* mutation among 56 clinically diagnosed MODY patients.

In the current study, a 40-year-old male was identified with a novel V177M mutation in the *PDX1* gene. *PDX1* mutations are a rare cause of MODY with pancreatic agenesis. However, milder forms of MODY have been reported among subjects with a *PDX1* mutation in individuals who were negative for the common MODY mutations.[21]

We have identified another novel missense mutation L92F in *HNF1B* gene in a 27-year-old female. Unlike the typical forms of MODY5 who present with genitourinary anomalies and variable severity of the renal phenotype,[22] abdominal imaging was negative for such lesions.

M033 is a 14-year-old boy with a history of polyuria, polydipsia, and weight loss who was initiated on insulin since diagnosis. He did not have ketosis and was negative for GAD and IA2 antibodies. In this subject we have identified a novel R31L *PAX4* mutation in a highly conserved region with a GERP score of 5·73. His grandmother and mother were diagnosed with diabetes at the age of 30 years, whereas his father was diagnosed at the age of 45 years. A similar phenotype has been reported with Arg37Trp[23] and c.374-412 del39[24] mutations in this gene.

In the present study using NGS with 314 and 316 Ion chips, three to ten samples were multiplexed and sequenced at an average depth of × 501 with 96·6% of the target sequenced at a minimum coverage of × 20 (Figures S1 and S2). We have validated this protocol by comparing Sanger sequencing and Ion PGM data of the HNF1A gene in ten samples (Table S1). All single nucleotide variants (SNV) identified by the Ion Torrent PGM were found to be concordant with Sanger sequencing.

Discussion

Until recently, genetic studies among individuals of Asian Indian origin related to MODY have been limited to the screening of *HNF1A, HNF4A* and *GCK* genes.[20,25,26] In the current study we have performed a comprehensive MODY genetic screening using next-generation sequencing. To the best of our knowledge, this

is the first report of *PDX1*, *HNF1B, NEUROD1* and *PAX4* mutations in the Asian Indian population.

In the present study with a parallel NGS approach, we have identified a wide spectrum of MODY mutations with variable clinical phenotypes. It has been previously reported that with extended inclusion criteria,[2] the mutation-positive rate was around 10–20%.[2] In previous studies from Indian population, the mutation-positive rate has been less than 10% when tested for the three commonly screened MODY genes; whereas a minimum of around a 30% mutation-positive rate was reported from Western laboratories in subjects who met the classical criteria.[27] However, it is well documented that about 50% of the mutation-positive MODY subjects do not meet the classical guidelines[27] for genetic testing. Therefore, in the present study with the extended criteria[2] and a ten gene panel, a diagnostic yield of 19% was obtained. Furthermore, even in this study 45% (5/11) of the subjects with an age more than 25 years would have been missed if the classical criteria[27] was used for diagnostic sequencing. The remaining majority of the mutation negative subjects (81%) may be attributed in part due to a lower prevalence of the commonly reported genes in the Asian Indian population or the presence of other rarer forms or undetermined MODY gene (MODY X) mutation.

NEUROD1 *and* PDX1 *mutations*

NEUROD1 mutations have been reported in six families till date.[12,13] In this study, four patients were identified to have *NEUROD1* mutations of which two were novel (in M18 & M30) and a reported mutation was present in M26 and M47. Interestingly, M26 and M30 had an additional E224K *PDX1* mutation, which has been reported in an Indo-Trinidadian family cosegregating with early-onset diabetes.[14] In these subjects with coexisting NEUROD1-PDX1 mutations, we performed an oral glucose tolerance test in M30 and a mixed meal challenge test in M26; both these subjects have shown a significant decline in insulin release when compared with T2D and control subjects (Table 2). Studies have shown that *PDX1* and *BETA2/NEUROD1* participate in a transcription complex formation that is essential for short-range DNA looping and insulin gene expression.[28] Therefore, it would be of interest to study the role of these digenic mutations in the transcriptional complex formation, insulin gene looping and its expression.

In M47, a 35-year-old obese (BMI: 39·7 kg/m^2) female patient, was diagnosed with diabetes at the age of 24 years and for the past two years she is being treated with insulin for glycaemic control. In this patient we have identified the H241Q *NEUROD1* mutation, which has been previously reported in families with obesity.[13] However, this mutation was also present in M26 who was not obese but had the coexisting E224K *PDX1* mutation.[14] Although the clinical phenotype of some *NEUROD1* variants remains unclear,[12,13] we hypothesize that additional diabetogenic factors conferred by increased body weight, as in M47, or a digenic mutation, as in M26 and M30, may be required for translation of beta-cell dysfunction into diabetes at an early age. A similar phenomenon of increased body weight required for translation of β-cell abnormalities into diabetes has been previously reported with B lymphocyte tyrosine kinase (*BLK MODY* 11) gene mutations.[29] Indeed, the mean BMI of the subjects who were MODY positive in our study was on the higher side by Asian Indian standards,[11] which highlights the necessity of more broad-based screening of MODY, and that patients who are not necessarily lean may have genetically diagnosable MODY.

NGS in clinical molecular diagnostics

Recent studies which have compared the performance of high-throughput bench-top next-generation sequencers Ion Torrent PGM (Life technologies) and Miseq (Illumina, San Diego, CA, USA) have shown that both platforms provide accurate detection of genetic variants with adequate coverage metrics.[30] In our study, using multiplex PCR-based enrichment for 10 MODY genes, 96·6% of the target was sequenced adequately on Ion Torrent PGM at a minimum of × 20 coverage. In a similar NGS approach using exon capture assay for 29 genes implicated in monogenic diabetes, 98% of target bases were sequenced with more than a × 20 coverage on Hiseq 2000 (Illumina).[31] Using the multiplex PCR enrichment strategy, the high GC rich targets were also enriched and the amplification of the complete target could be analysed before proceeding for sequencing. Therefore, we could achieve more than a × 20 coverage even

Table 2. Comparison of insulin levels in maturity onset diabetes of the young (MODY) patients with digenic mutation, T2D and normal controls

Time	0 h			1 h			2 h		
Subjects	GLU	INS	C PEP	GLU	INS	C PEP	GLU	INS	C PEP
M30 (NEUROD1 & PDX1)	9·7	18·2	0·52	16·7	33·1	1·32	15·6	71·4	1·39
T2D (N = 10)	7·3	48·0	–	13·5	924	–	11·9	744	–
Control (N = 23)	5·5	33·0	–	9·2	840	–	6·7	552	–
M26* (NEUROD1 & PDX1)	13·3	25·3	0·7	17·6	155	1·31	17·0	120	1·31

GLU, Plasma Glucose levels (mM); C-pep, C-peptide (nmol/ml); INS, Insulin (pM); T2D, Type 2 diabetes.
*Mixed meal challenge test.

in the GC rich regions (*PDX1* exon 2 and *KLF11* exon 1). All identified mutations and rare variants were confirmed using Sanger sequencing. The Ion Torrent NGS data was 100% concordant with Sanger sequencing data in identifying single nucleotide variants (Fig. 3 and Table S1).

Implications of NGS-based MODY genetic diagnosis

At present, Sanger sequencing of three MODY genes [*HNF1A, GCK and HNF4A*] is expensive with a turnaround time of 40 days for reporting a mutation.[32] Utilizing the (2GDMODY protocol) Ion Torrent PGM, genetic screening of a panel of ten MODY genes, with a flexibility of sequencing 3–10 samples utilizing Ion 314 or 316 chips, it would cost four times less, with a turnaround time of 30 days. Furthermore, the specific variants identified by NGS can be confirmed by Sanger sequencing.

A confirmed genetic diagnosis of MODY is likely to have important prognostic and therapeutic implications.[1] It may help in streamlining treatment and enable physicians to avoid insulin therapy in a majority of the patients with confirmed *HNF1A* and *HNF4A* mutations.[33] Identification of family members with specific mutations will help in facilitating genetic counselling, regular monitoring and initiation of lifestyle modifications and other appropriate therapy at an early stage.

A recent study that had utilized NGS-based screening of a 29 gene panel, implicated in monogenic diabetes[31] has shown a mutation detection rate of 15%. In addition, the same study has shown that 6 of 14 newly identified MODY mutations were in genes that were not screened due to the absence of the characteristic features of genetic subtypes.[31] It is a well known fact that the subjects with the same form of MODY and also families carrying the same MODY mutation present with a variable clinical phenotype.[4,34] Furthermore, the identification of novel digenic NEUROD1-PDX1 mutations in this study along with the recent reports of coinheritance of *HNF1A* and *HNF4A* mutations in siblings[35] and coexistence of digenic *HNF1A* and *GCK* mutations,[36] projects the need for parallel multi-gene testing in MODY. These finding could have been missed if we had followed conventional sequential screening methodology. Therefore, we suggest a comprehensive genetic screening in patients who have an autosomal dominant inheritance with young onset diabetes (<35 years) in the absence of β-cell autoimmunity.

Limitations of the study

The family studies and investigations related to insulin secretory capacity of the mutation-positive subjects were possible in only a subset of patients. Further family and functional studies are required to confirm the role of these novel 5′ UTR and nonsynonymous coding variants in the pathogenesis of MODY.

Currently, due to a high rate of indels in homopolymer stretches,[30] Ion Torrent PGM could miss true indels and therefore require multiplex ligation-dependent probe amplification (MLPA) assay to detect deletions or duplications. However, with recent improvements in the sequencing chemistry, it is expected to significantly reduce indel errors at the homopolymer stretches.

Conclusion

NGS provides a rapid, cost-effective and accurate molecular method for comprehensive parallelized genetic testing of MODY. We have identified a high frequency and a novel digenic mutation pattern involving *NEUROD1* and *PDX1* genes. These findings differ from earlier reports and may have been missed by selective sequential genetic testing. Following the FDA approval of one of the NGS technologies, future studies and collaborative efforts are necessary to validate and utilize these approaches to make genetic diagnosis affordable for patients with young onset diabetes.

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Contribution

AC and NT designed the study; AC designed and standardized the experiments; AC, DV & MV performed, acquired, and validated the data; MDM, PV, VN, SM, TVP, HSA, NT were involved in patient recruitment and acquiring clinical data; AC and NT analysed the data; AC, MDM, SV & NT wrote the manuscript. All the authors were involved in manuscript review and revision.

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