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A NOVEL MUTATION IN THE HEPATOCYTE NUCLEAR FACTOR-1 A GENE AMONG MODY EGYPTIAN PATIENTS

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ABSTRACT

The hepatocyte nuclear factor 1α (HFN1α) is a member of the nuclear receptor superfamily that regulates genes which play a critical role in glucose metabolism. A genome-wide association studies showed that mutations in the human HNF1α gene cause a maturity onset diabetes of young (MODY3). Accordingly, HNF1-α mutants caused □-cell dysfunction in MODY3 is caused by loss of function mechanisms like reduced DNA binding, impaired transcriptional activation, and defects in subcellular localization (Ghosal *et al.*, 2012). This study was done to determine genotype characteristics of eight Egyptian patients with HNF1α variants caused MODY3 type and to examine the influence of HNF1α mutation type, or location on clinical phenotypes. Blood of eight child Egyptian patients (three females and five males) aged 13.6 years ± 6.18, and their age at diagnosis was 5.5 years ±3.4 were analysed to detect the possible mutations of the HNF1-α gene. Their clinical investigations indicating that, FBS was 179 mg/dl ±54.2, PPS was 265.6 mg/dl ± 74.7, C-peptide was 2.2 pmol ±0.38, HbA1c ≥ was 10.4% ±2.9, HOMA-IR (0.96 ± 0.366) and BMI was 26.26± 5.79. Genomic DNA was extracted then polymerase chain reaction and DNA sequence analysis were performed accordingly for 10 entire coding exons with specific primer sets. A total of 22 mutations prevailed; 4 were previously known mutations (I27L, E90V, E105G and S487N), in addition to 8 silent mutations (G288G, P379P, L459L, G323G, L341L, V344V, L369L and P488P) were detected. Frameshift mutation (c.1189delT23) caused a silent mutation (S388S) and created a stop codon after 23 amino acid producing a truncated protein with 411 amino acids (M412X). The deduced protein missed 190 amino acids from carboxylic terminal. Moreover, nine missense novel mutations were first identified among Egyptian patients; all of them were located in Exon 5 (P325D, P334S, V342G, T346P, P347S, S359R, S361N, L362Q, L363W). It was observed that maximum number of variations were existed in exon 5 of HNF1-α, which may be referred to "Mutational Hotspot" as previously reported by (Ghosal *et al.*, (2012). The position of the mutation relative to the functional domains of HNF1-α, obesity state and HOMA-IR also plays a role in the severity of MODY3 disease.

KEYWORDS: Hyperglycemia, MODY, HNF1α, mutational analysis.

INTRODUCTION

Maturity-onset diabetes of the young (MODY), which comprises between 1 to 5% of all the cases of diabetes, is diagnosed as a monogenic non-ketotic form of diabetes mellitus and often misdiagnosed as type 2 diabetes. Actually, it is distinct from a typical type 2 diabetes in that its inheritance is autosomal dominant, a prominent family history of diabetes in three or more generations, asymptomatic to mild hyperglycemia, non-obese patients and characterized by early clinically onset usually occurs at less than 20 years of age (Vaxillaire and Froguel 2006). MODY has numerous subtypes, depending upon the gene that is involved, at least 13 genes have been identified with mutations that cause MODY (Kim 2015) based on the sequence of genes identified were referred to as MODY 1-13. Heterozygous mutations in those genes that coding for various transcription factors involved in the fetal development of β-cells development as well as those regulating the maturation and maintenance of β-cell function caused wide clinical and genetic heterogeneity within and among the different MODY subtypes. The different transcription factors involved in fetal pancreatogenesis include hepatocyte nuclear factor 1-α (HNF-1α), HNF-4α, HNF-1β, IPF-1 (Insulin Promoter Factor-1), Neuro-D1 (Neurogenic Differentiation Factor-1) and Pax 4 and 6 (Paired Homeobox), in addition, mutations in enzymes directly or indirectly involved in glucose sensing of the β-cell are

the main cause of MODY (Babenko *et al.*, 2006). In fact, mutations in transcriptional activator HNF-1α gene (MODY3) are the most common among other subtypes, which accounts 52% in studies from UK and from many European countries (Kavvoura and Owen 2012).

HNF1α is a homeodomain function containing transcriptional activator that regulates the tissue specific expression of multiple genes, especially in pancreatic islet cells, kidney, gut and in liver. It has a crucial role in regulation of transcription of insulin gene in the mature β-cell as well as the glucose transporter GLUT2 (Galán *et al.*, 2011). Therefore, mutations in coding and non coding region of HNF1α gene caused subtype of MODY3. It is characterized by a severe insulin secretion defect, a retained sensitivity to sulfonylureas, a decreased renal threshold for glucose reabsorption and the occurrence of liver adenomatosis (Christine *et al;* 2008). The clinical expression of MODY3 is highly variable from one family to another or even within the same family (Rose *el al.*, 2000).

HNF1α is homeodomain which consists of ten exons, coding 631 amino acids and has been subdivided into three functional regions: an amino-terminal dimerization domain (residues 1–32), a DNA binding motif containing an atypical homeodomain (residues 203– 276), and a carboxyl-terminal transactivation domain (residues 281–631) (Mendel *et al.*, 1991). More than 300 different MODY3-causing mutations have been found in both the coding sequence and promoter of HNF1α. These mutations include gene deletion, frame shift, missense, nonsense and splice site mutations in more than 700 families (Bellanne-Chantelot *et al.*, 2008).

Hyperglycemia due to mutations in HNF-1α has been found to be well controlled with sulfonylureas for a number of years (Fajans *and Brow* 1993). It is therefore important to characterize the different types of MODY and to distinguish it from other types of diabetes, because the treatment protocols for achieving optimal glycaemic control are specific for each type of MODY. The aim of study was to characterize patients classified as HNF1α-MODY and investigate whether mutation type (missense, nonsense and frameshift), or functional domain of mutation (dimerization-, DNA binding- or transactivation domain) was related to clinical phenotype. In addition, to determine the relation between the molecular characteristics of the HNF1α mutations severity of the disease.

MATERIAL AND METHODS

Patients and subjects

A Total of 200 Egyptian hyperglycemic patient age from 3-18 out of 2000 patient admitted to endocrinology clinic paediatric children hospital Ain- Shames University Cairo Egypt from a period 2010-2013 were selected. According to the clinical criteria and via questionnaires, they were classified into two groups. Group (1) of 192 insulin-dependent diabetes mellitus (IDDM) type-1 with C-peptide ≤ 0.2 nmol/L, and group (2) of 8 Non-insulin-dependent diabetes mellitus (NIDDM) diagnosed as MODY (The diagnostic criteria followed those of the 57th ADA Conference held in Boston in June 1998), where C-peptide ≥ 2.0 nmol/L. This study includes only group-2 (three females and five males) aged 13.6 ± 6.18 years, and their age at diagnosis with diabetes was 5.5 years ±3.4. Four of them had no family history of diabetes (table 1). All patients gave written informed consent for the screening for all exons mutations and polymorphism. Alongside with clinical diagnosis all information on patients family history of diabetes as first or second degree relative was diabetic, current age, age at diagnosis, type of treatment received.

Table 1: Clinical features and characteristics of HNF1α-MODY patients.

Patient' data	1	2	3	4	5	6	7	8
Gender	female	male	male	male	female	male	female	male
Birth 'date (m/y)	10/1991	3/2001	4/2003	5/1991	8/2004	8/1994	10/2007	4/1996
Admission 'date (m/y)	11/2000	3/2005	8/2009	5/2010	4/2007	7/2007	3/2010	4/2004
Diabetic family pedigree	---------	mother	Mother, father	sister	brother	--------	----------	-------

Method

Serum biomarkers for MODY, C-peptide assays was carried out on peripheral serum by biovision C-Peptide ELISA Kit, glycosylated haemoglobin (HbA1c) was measured by GHbA1c ELISA kit, overall picture of fasting and 2h postpendial blood sugar levels was measured by the method of glucose oxidase kit (Barham and Trinder 1972). Calculated HOMA-RI was carried out according to Matthews *et al.*, (1985) method and body mass index (BMI) was measured according to Ford *et al.*, (2010) were also determined

Genomic DNA isolation: From each subject, a blood sample (2 mL) was collected into a tube containing EDTA. Genomic DNA was extracted from the whole blood using a genomic DNA extraction kit (Promega, Madison, WI, USA). Genomic DNA was isolated using standard phenol-chloroform method of isolation and the quality of the DNA checked by spectrophotometric analysis (Sambrook *et al.*, 1989).

Polymerase chain reaction (PCR): Amplification of 10 exons of the HNF1-α gene was carried out with 100 ng of extracted DNA. The reaction mixture in 50 µl

consisted of 10 mM Tris-HCL (pH 8.3), 50 mM KCL, 2.5 mM MgCl2, 2.5 mM deoxyribonucleoside triphosphates, 1 U Taq DNA polymerase (MBI Fermentas) and 20 µmol of 10 different sets of primers specific for each exon. The reactions were performed at various annealing temperatures specific for each exon as represented in table-2 in a thermo cycler (Perkin Elmmer. USA). The cycling conditions was 15 min at 95°C followed by 30 cycles consisting of 30s at 94°C, 30s at average of 62°C, and 45s at 72°C. Amplification of all DNA fragment of the HNF1-α, gene was done using the upstream and downstream primers listed in table 2 (Boutin *et al*., 2001). Amplified fragment products were run on agarose gel electrophoresis (Sambrook *et al.*, 1989), and following electrophoresis, the samples were purified using commercial kits (Roche).

Table 2: PCR primer sequence sets for the HNF-1α gene (Boutin *et al*., 2001).

Exon	Primer set	Primer Sequences	Primers size (mer)	Tm (°C)	Amplified Size (bp)	Position on DNA (g)
1	Forward primer	5`-GGCAGGCAAACGCAACCCACG -3`	21	56	349	5,001..5,349
	Reverse primer	5`-GAAGGGGGCTCGTTAGGAC-3`	19			
2	Forward primer	5`-CATGCACAGCCCCACCCTCA-3`	20	60	200	15,088..15,287
	Reverse primer	5`-CTTCCAGCCCCCACCTATGAG-3`	21			
3	Forward primer	5`-TGAGAGTGGCCAGTACCC-3`	18	55	263	19,775..19,961
	Reverse primer	5`-CAAACCAGCACTGTTTCC-3`	18			
4	Forward primer	5`-CAGAACCCTCCCCTTCATGCC-3`	21	58	242	20,419..20,660
	Reverse primer	5`-GGTGACTGCTGTCAATGGGAC-3`	21			
5	Forward primer	5`-GGCAGACAGGCAGATGGCCTA-3`	21	60	152	22,517..22,668
	Reverse primer	5`-GCCTCCCTAGGGACTGCTCCA-3`	21			
6	Forward primer	5`-TGGAGCAGTCCCTAGGGAGGC-3`	21	58	202	22,796..22,997
	Reverse primer	5`-GTTGCCCCATGAGCCTCCCAC-3`	21			
7	Forward primer	5`-GGTCTTGGGCAGGGGTGGG-3`	19	55	347	23,729..23,920
	Reverse primer	5`-CTGCAATGCCTGCCAGGCA-3`	19			
8	Forward primer	5`-GAGGCCTGGGACTAGGGCTGT-3`	21	64	229	25,523..25,644
	Reverse primer	5`-CTCTGTCACAGGCCGAGGGAG-3`	21			
9	Forward primer	5`-CCTGTGACAGAGCCCCTCACC-3`	21	62	287	25,738..25,882
	Reverse primer	5`-CGGACAGCAACAGAAGGGGTG-3`	21			
10	Forward primer	5`-GTACCCCTAGGGACAGGCAGG-3`	21	58	251	27,320-.28,767
	Reverse primer	5`-ACCCCCCCAAGCAGGCAGTACA-3`	22			

Direct sequencing of the PCR amplified products: It was performed using an ABI Dye Terminator Cycling Sequencing kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions (Sanger *et al*., 1992), followed by analysis on an ABI3700 DNA sequencer (Applied Biosystems). Data analysed was carried out by mega6 software. Novel mutations were submitted to GenBank NCBI.

Analysis of data: Sequencing results are used for nucleotide blast on http://blast.ncbi.nlm.nih.gov/ Blast (National centre for Biotechnology) to make alignment with the GCDH gene normal strand by using its accession number NM_000159.3 to detect new mutations. All detected mutations submitted on https://mutalyzer.nl/name-checker to detect the effect of the mutation on the amino acids sequence of the GCDH peptide and detect the missense mutations. The mutalyzer gives also the expected protein sequence of the missense mutations and nonsense mutations. The amino acid sequence are used then to detect the 3-dimension structure of the whole enzyme and compare it with the normal GCDH. The amino acid sequences submitted to http://raptorx.uchicago.edu/. To detect the expected 3D protein structure. The mutation nomenclature followed the recommendations of the Human Genome Variation Society.

Statistical analysis: All the results were expressed as Mean ± S.D. Comparisons between the control and the treated groups were done using the student's t-test. Differences were considered statistically significant at $p<0.05$. Change % was also calculated for all values using SPSS 16 software. If a specific genetic defect in HNF1α was found, patients were registered in the database as HNF1a MODY to specify how HNF1a-MODY diagnosis was confirmed and to provide detailed genetic data on the HNF1α.

RESULTS

Description of the study cohort

A Total of 200 Egyptian hyperglycemic patient aged from 3-29 years out of about 2000 patients admitted to endocrinology clinic in paediatric hospital, Ain- Shames University, Cairo, Egypt from a period 2010-2013. They were clinically diagnosed clinically as diabetic patients, and divided into two groups. Group (1) including 192 insulin-dependent diabetes mellitus (IDDM) patients or T1 DM with C-peptide ≤ 0.2 nmol/L; and group (2) with

8 Non-insulin-dependent diabetes mellitus (NIDDM) or T2 DM with C-peptide ≥ 2 nmol/L as enrolled with fulfilling stringent MODY criteria, males (5/8; 62.5%) and females (3/8; 37.5%) with age 13.6 years ± 6.18. Chronological age was 5.5 years ± 3.4, and disease duration was 5.8 years ± 2.8 years. Treatment with diet may be adequate initially, but there is an increase in hyperglycemia. Patients 7 eventually was requiring sulfonylurea tablets treatment as physician recommended.

Characteristics of patients with HNF1α mutations

According to **Ellard *et al.*, (2008)** recommendations for NIDDM patients, the early onset (usually before 25 years of age), of severe and progressive hyperglycemia, concurrently affecting other family members (with at least one and ideally two, family members affected, a family history of diabetes) and together monogenic autosomal dominant inheritance. Patients mostly presented with hyperglycaemia (92.6%) and typical symptoms including polydipsia, polyuria and glucosuria at diagnosis were documented (multiple symptoms per patient were possible). The sample consisted of sex unrelated patients and one sibling patient suffering from medicinally untreatable diabetes. A family history (first degree relative; both parents, only mother and siblings representing 1/8. 1/8; and 2/8 respectively) of diabetes was reported for 50% of subjects with HNF1α mutations, and 50% without any family history for second or third relative (Table 1).

Patients mostly presented with upper normal to moderate hyperglycaemia (6/8; 75%) and typical symptoms including polyuria (n=2) and glucosuria (n=5) at diagnosis were documented. Some of the biochemical features of these patients represented in Table 3. They were identified by clinical feature of fasting plasma glucose (FBS) 180 mg/dl ±54.32, 2-h glucose level (PBS) after a 75-g of oral glucose tolerance test (OGTT) 265.6 mg/dl ± 74.7, C-peptide (2.18 nmol/L ± 0.398), HbA1c ≥ 8.93% ± 3.9. HOMA-IR stands ranged from 0.65 -1.6 (0.96 ± 0.366) and these patients were slightly overweight to obese where their body mass index (BMI) was 26.26 Kg/m^2 ± 5.79. Patients 5, 6, and 7 are high HOMA-RI reflecting that they are in high risk for treatment with insulin soon or later. Lifestyle intervention not reported for insulin or with oral antidiabetic drug (OAD) for seven patients, treatment of one patient (12.5%) through oral antidiabetic sulfonylurea drug (patient 7) reported as recommended by doctors. All patients gave written informed consent the screening for all exons. Clinical characteristics of studied subjects with HNF1α mutations were presented in Table 3.

Table 3: Characteristics of HNF1α - MODY3 patients.

Parameters/ Patients	1	2	3	4	5	6	7	8
Fasting blood sugar FBS (mg/dl)	122	135	126	190	235	190	275	170
Postprandial blood sugar PPS (mg/dl)	220	178	207	250	420	290	300	260
C-peptide nmol/L	2.3	2.4	2.1	1.6	2.1	2.8	2.4	1.7
Hbc1A %	10.4	11.6	13	9,5	10.1	8.8	7.2	10.9
HOMA-IR	0.69	0.69	0.65	0.75	1.21	1.31	1.6	0.71
BMI* (Kg/m^2)	18.5	28.9	22.9	30.9	32.1	33.5	23.4	19.9

FBS= Fasting blood sugar, PBS= Postprandial blood sugar, Hb1cA= glycosylated Haemoglobin, HOMA-IR= Homeostatic model assessment for insulin resistance (Matthaws *et al.*, 1985), BMI= Body mass index.
BMI <25Kg/m^2 thinness; > 25 Kg/m^2 overweight; >30 Kg/m^2 obese for children 5-19 years old (Ford *et al.*, 2010)

Genomic DNA was extracted from blood samples with commercial kits (Qiagen; USA). PCR amplification was done with specific ten sets of oligonucleotides for the flanking regions of 10 exons of *HNF1*α gene (Boutin *et al*., 2001). Following electrophoresis, the samples were purified, and sequenced. Molecular analysis of the amplified fragments of 8 patients revealed twenty-two different mutations distributed throughout the HNF1α gene that represented in forty eight variations. It was clear that, many mutations repeated in many patients as well same mutation repeated in different patients (variants). One frameshift deletion in one variant (1/22; 4.6%), eight synonymous in 23 variants (8/22; 36.4%), and thirteen missense mutations in 24 variants (13/22; 59%). All mutations were existed in exons 1, 4, 5, 6, and 7. Fourteen mutations were heterozygous (63.6%) and 8 mutations were homozygous (36.4%). One missense mutation located (4.6%) in the dimerization domain, 2 missense mutations in the DNA binding (9.2%) in addition to one frameshift mutation (4.6%), and 18 other types of mutation were localized in the transactivation domains (81.6%), which both affected the structure of largest domains on the HNF1α. Mutations were located throughout the coding region of HNF1α; mostly localized in exon 5 (13/22; 59%) and to the lower extent exons 1, and 7 (each represents 3/22; 13.6%), in addition to 2 mutations at exon 6 and one mutations at exon 4. However, no mutation was detected in exons 2, 3, 8, 9, or exon 10.

Classification of HNF1α mutations

Generally, silent mutations are usually classified as allelic polymorphisms, which may be neglected in analyses of wider interest because they are considered to

be neutral. Cartegni *et al.*, (2002) compiled more than 20 studies reporting specific points of synonymous mutations within coding regions associated with altered splicing, which in turn led to the exclusion of certain exons. In this study, none of the synonymous variants found in exons of the *HNF1α* gene was associated with known donor sites for splicing. Our study identified eight silent mutations (G288G, P379P, L459L, G323G, L341L, V344V, L369L and P488P) in 23 variants among 8 patients distributed in 5 exons. Their clinical characteristics were not significantly different from other patients with HNF1α missense mutations.

Our attention was focused on coding regions of HNF-1α where diabetes-associated missense/frameshift mutations cluster, because single amino acid substitution or shifted are informative measures of protein function. Apart from 8 silent mutations detected, 4 out of 13 missense mutations (30.8%) were previously identified and described in different studies (Holmkvist *et al.*, 2006; Morita *et al.*, 2015). These included one mutation (I27L) in dimerization domain (exon 1); two mutations (E90V, and E105G) in the DNA binding domain (exon 1); one mutations (S487N) in the transactivation domain (exon 7). Remaining nine missense base substitution mutations were novel mutations in our study representing 69.2% which indicating that these kind of mutations are specific to the Egyptian population and not previously reported elsewhere. They all were distributed in exon 5; c.973C>G; p.P325D, c.1000C>T; p.P334S, c.1025C>T; p.V342G, c.1036A>C; p.T346P, c.1039C>T; p.P347S, c.1077C>A; p. S359R, c.1082G>A; p.S361N, c.1085T>A; p.L362Q, and c.1087C>T; p.L363W (table 4). One silent mutation in exon 6 was happened due to base deletion mutation at position 1189 (-T) resulted in stop at position M412 codon (M412X) after 23 amino acid (c.1189delT; p.S388S*23) to produced truncated functionless protein which accounted also as a novel mutation.

Table 4: Missense mutations in the HNF-l *α* gene identified in MODY3 among studied Egyptian patients.

Change at the genomic level	Change at the cDNA level	Change at the protein level	Exons	Patient no	Frequency	Novel mutations
g. 5102 C>G	c. 74 C>G	p. 27 I-> L	E1	2,3,8	3	known
g. 5292 A>T*	c. 292 A>T*	p. 90 E->V		6,7	2	known
g. 5339 A>G	c. 339 A>G	p. 105 E>G		4	1	known
g. 5982 G>T*	c. 982 G>T *	p. 325 P->D	E5	6	1	Novel
g. 5907 T>C*	c.1007 T>C*	p. 334 P->S		6,7	2	Novel
g. 6059 T>G*	c.1059 T>G*	p. 342 V->G		6	1	Novel
g. 6100 C>A*	c.1100 C>A*	p. 346 T->P		6,7	2	Novel
g. 6105 A>G*	c.1105 A>G*	p. 347 P->S		7	1	Novel
g. 6110 A>G*	c.1110 A-G*	p. 359 S->R		5,7	2	Novel
g. 1629 G>T*	c.1129 G>T*	p. 361 S->N		3,6	2	Novel
g. 1789 G>A	c.1289 G>A	p. 362 L->Q		4,7	2	Novel
g. 1983 G>A	c.1483 G>A	p. 363 L->W		6,7	2	Novel
g. 6189 delT	c. 1166 delT23	p. 388 S ->S	E6	3	1	Novel
g. 6460 G>A	c.1460 G>A	p. 487 S->N	E7	1,6,8	3	known
total		14 mutations			25 variants	

* Heterozygous mutations

Three-dimensional structures have been reported for the dimerization domain and the homeodomain. The dimerization domain forms a four-helix bundle, with each identical promoter contributing two α helices separated by a turn (Narayana *et al.*, 2001). The three α helices of the HNF-1α homeodomain are superimpose able on the three helices of other homeodomains, although there is an extended loop between the second and third helices of HNF-1α relative to the canonical homeodomain fold (Schott *et al.*, 1997). Structures of HNF-1α bound to promoter DNA has been reported by mutalyzer programme.

DISCUSSION

HNF-1α is a homeodomain-containing transcription factor expressed in liver, kidney, pancreas and gut. HNF-1α contains three functional domains; the N-terminal dimerization domain (residues 1–32), the DNA binding domain, (spanning residues 91–281), and a C-terminal transactivation domain (residues 282–631). Mutations in HNF1α are a common cause of monogenic MODY3 diabetes (Nogaroto *et al.*, 2011), therefore, functional studies of a relatively small number of HNF-1α mutations, usually focused on their effects on expression of a single target gene, have shown that diabetes can result from loss-of-function or dominant-negative effects (Yu *et al.*, 2015). The clinical phenotype of MODY3 is variable from one family to another and heterogeneous within each family. This variability has been explained by environmental and/or additional genetic factors. Additionally, the type and/or position of the mutation appear to modulate the age of diagnosis (Bellanne-Chantelot *et al.*, 2008).

Regarding a family history, about 50% of all subjects indicated that mothers of both patients (2 and 3) with HNF1α mutations were more likely to inherit diabetes to their children than other first-degree relatives. Predominance of maternal diabetes among patients with diabetes has been previously reported (Kim *et al.*, 2003). In this study sample, mothers were more often diabetic when patients carried HNF1α mutations (25%). Data on diabetes during pregnancy were not available for further analysis of possible links to predominance of diabetic mothers in this group. There were similar percentage as diabetic siblings (25%; patients 4 and 5) when patients carried HNF1α mutations. Apart from genetic predisposition, environmental factors, age and obesity, may play an influential role in disease manifestation in patients carrying HNF1α mutations (Li *et al.*, 2016).

The majority of patients presented with hyperglycaemia at disease onset and specific symptoms including polyuria and glucosuria (4 patients) were reported for 50% of patients. Remaining four patients 50% was diagnosed through a first degree relative who had been identified as NIDDM and most probably of HNF1α-MODY. The variability of HNF1α-MODY clinical phenotype is further reflected by the fact that more than one quarter of patients with mutations were asymptomatic.

Factors, such as severe failure of glycemic control in patients, placed in evidence by fasting and postprandial testing, obesity and insulin resistance in response to glucose, are requisites for triggering the onset of diabetes (Hegele *et al.*, 1999). The marked presence of polymorphisms already associated with T2DM that present in the gene responsible for MODY3 permits the classification of these patients as typical MODY3 patients (Glamoclija and Jevric-Causevic 2010). Furthermore, the perceptibly constant presence of overweight patients in this study could be an indication of an even more complex relationship between the development of obesity and the polymorphism found, especially as regards patient 1, who carried two well identified the variants (I27L and A98V), and who, at the age of 19 and was overweight.

Sanger sequencing is a widely used genetic diagnostic platform for identifying MODY and is the gold standard sequencing platform to test single-gene genetic disorders. This sequencing technique has been reported to be >99% sensitive to detect a heterozygous base substitution in MODY genes. Many countries are now offering molecular diagnosis to young people with diabetes before a therapeutic decision can be made. Additionally some of them have established monogenic diabetes registries (Ellard *et al.*, 2008).

We examined 8 suspected MODY3 diabetes patients labelled HNF1α-MODY, diagnosed in Ain Shams Hospital, Genetic research unit, Cairo, Egypt. A total of 22 different HNF1α mutations were detected among 48 variants throughout the HNF1α gene, almost half of variants were silent mutations (8/23). Other 25 variants were detected in 14 different missense/frameshift mutations (14/25). Like in other studies (Pearson *et al.*, 2003), disease phenotype among silent and missense mutations was highly variable in the cohort. Missense mutations were frequently distributed in exons 1, 5, 6, and 7 in the study sample. The distributions of mutations indicated that missense errors were more abundant in dimerization domain, DNA-binding domain and transactivation domain while frameshift mutation was located only in the transactivation domain. This distribution is consistent with other studies, which show that the transactivation domain is more accommodating of mutations causing changes in protein structure than the DNA-binding domain (Harries *et al.*, 2005; Ellard and Colclough 2006). We investigated the effects of HNF1α mutation types, and domain occurrence on age at disease onset and clinical phenotype of HNF1α-MODY. It is clear that patient's age at onset of disease did not vary depending on mutation type or domain localization.

Analysis of missense mutations by MODY3 patients

Out of 22 mutations, there were 13 (59%) missense, 8 (36.4%) silent and 1 (4.6%) frameshift mutations were detected in the sample The majority of missense mutations (9/22; 40.9%) in exons 5 only, and remaining missense mutations were located in exons 1, and 7 (3, and 1 representing 13.6%; and 4.5% respectively of missense). However, one frameshift mutation was localized in exon 6 (4.5%). Only four mutations out of fourteen were identified previously and the remaining ten mutations in this study sample were novel and undescribed before.

Regardless of silent mutations, it is interesting to notice that each of 8 patients in our study contain a compound mutations (more than one mutation among ten exons). Patient 1 has a homozygous missense mutation reported before c.1483G>A (p.S487N) in exon 7. Patients 2 has two compound homozygous mutations, one c.102A>C (p.I27L) in exon 1 and one mutation in exon 7 c.1483G>A; p.S487N. Patients 3 has a unique novel homozygous frameshift mutation c.1189delT23.and three other novel heterozygous mutations; p.V342G, p.S359R and p.S361N. Patient 4 contains two previously identified homozygous missense mutations (p.E90V and p.E105G) as well as one heterozygous mutations p.T346P. Patient 5 has three mutations (p.P325D, p.P334S, p.P347S). Patient 6 has two homozygous mutations p.I27L, p.E90V, and two novel heterozygous p.P325D, p.V342G, which appears to be the second common mutation in Egypt. Patient 7 has two novel heterozygous mutations (p.T346P, and p.L362Q) in addition to one previously identified homozygous mutation (p.S487N). Patient 8 has two previously identified homozygous mutation (p.I27L, p.S487N). Holmkvist *et al.*, (2006) regarded that in MODY3 patients, the main variants to be found in the HNF1 gene were I27L, A98V, G319S and S487N.

The molecular spectrum of 13 HNF1α missense mutations were analysed according to two criteria. First, mutations were classified into two groups; Homozygous mutations 4/22 (I27L, E105G, E90V and S487N), and heterozygous mutations 9/22 (P325D, P334S, V342G, T346P, P347S, S359R, S361N, L362Q and L363W). Second, mutations were analysed according to the three HNF1 functional domains. We found one known NH2-terminal dimerization domain 1/22 (I27L). Two mutations in DNA-binding domain molecular function 2/22 (E90V and E105G) and COOH-terminal transactivation domain 9/22 (P325D, P334S, V342G, T346P, P347S, S359R, S361N, L362Q, and L363W).

The four missense mutations (I27L, E90V, E105G and S487N) were previously precisely characterized also were identified in this work in eight variants among 8 studies Egyptian patients that clinically diagnosed as MODY3. The mutation (c.79 A>C) in exons 1 in three patients (2, 3 and 8) predicts a missense amino acid change (p. I27L). The I27L genotype explained 12.0% of the total variance in the insulin resistance index. It was significantly associated with insulin resistance, and β-cell function. This finding was rather expected, because this mutation in the HNF-1α gene lead to β-cell dysfunction in MODY3 patients (Ken *et al., 2013*). Similar results regarding genotype–phenotype correlation have been reported for this mutation type and location in previous studies (Frayling *et al.*, 2007). Additionally, many studies proved that patients with mutations affecting the DNA-binding domain were more often likely to use of insulin for treatment than mutations in the transactivation domain (Bellanne-Chantelot *el al*., 2008). Interestingly, the p.I27L polymorphism changes the tertiary structure of the protein from a loop to an -helix at this site (Harries *et al.*, 2006). Clinical analysis of patients carrying this mutation revealed that, they suffer from mild to severe hyperglycemia. However, the two youngest carriers of the HNF1α mutation (patients 2 and 8) were asymptomatic at the time of the study. Usually, MODY3 is a post pubertal disease; therefore, these subjects could be at either a pre-symptomatic or an early disease stage. Whereas the I27L was associated with a propensity to develop T2 DM, especially in overweight individuals (Holmkvist *et al.*, 2006). This relationship between BMI and I27Lwas also reported by Morita *et al.*, (2015), where 80% of the patients with this variant were also overweight. These data are consistent with our findings, wherein one of the two patients harbouring the polymorphism was overweight from the questioner sheet (table 4).

Mutations targeted the HNF-1 α dimerization domain (HNF1α) that affect a unique four-helix bundle. Through rearrangements of interfacial side chains, which binds the dimerization cofactor of HNF-1 (DCoH) causing reduction in its activator function (Michael *et al.*, 1997). Other two mutations (E90V and E105G) are missense mutations affecting the dimerization/DNA-binding domain have more severe functional consequences, such as impaired DNA-binding and protein stability (Bellanne-Chantelot *el al*., 2008).

Polymorphisms p.I27L and p.S487N together are associated with an earlier age of diagnosis (Harries *et al.*, 2006). Individual carrying both the p.I27L and the p.S487N polymorphism (patients 2, 3, 6 and 8) had an earlier age of diagnosis at 4 years of age. Our evolutionary analyses suggested that the polymorphisms p.S487N occur at conserved sites and are located to the transactivation domains (exon 7) of HNF1. The p.S487N mutation potentially affected the protein tertiary structure prediction and indicated that this missense mutation might play role in the development of T2 DM in patients examined in our study. The pathogenic mechanism of p.I27L and p.S487N is still unknown and needs further functional investigation.

We found the 9 novel and undescribed before mutations in exon 5, it appears to be the most significant exon hence screening studies should begin with this exon in our population. Therefore, exon 5 is likely to be rich in MODY3 mutations and may be referred to as the mutational hotspot. (Ghosal *et al*., 2012). Missense mutations (P325D, P334S, V342G, T346P, P347S, S359R, S361N, L362Q, and L363W), were located in COOH-terminal transactivation domain in the HNF-1α resulted in the greatest reduction in stimulation of transcription compared to wild-type protein. However, this domain probably consists of many elements that work in concert to give the full transactivation potential of the protein (Bjørkhaug *et al*., 2001).

The four novel missense mutations p.S359R, p.S361N, p.L362Q and p.L363W which have not been previously described before were identified in 5/8 patients representing 62.5%% under studying (patients 3, 4, 5, 6 and 7). These patients were clinically asymptomatic that located near to previously reported mutation p.S355X (Awa *et al.*, 2011). We however, did not have genetic reports of parents, but their localization increases the investigations to conclusively classify previously undescribed variants as novel mutations.

Five novel missense mutations p.P325D, p.P334S, p.V342G, p.T346P, and p.P347S, are all located in the Transcriptional activated domain and considered as a second mutational hot spot on exon 5 in this region area. The apparent lack of effect of these mutations is not expected, since there is a clear genotype/phenotype correlation in patients (6 and 7). However, these polymorphisms, which appear to be a MODY3 mutation, might be a risk factor for developing diabetes, since it was not detected in any of healthy subjects (personal communications).

Previous studies indicate that HNF1α nonsense-mediated mutations generating mRNA harbouring premature termination codons are likely to cause significant

reduction in protein levels and producing truncated protein which, is unlike missense mutations with milder functional consequences (Bellanne-Chantelot *et al.*, 2008; Nogaroto *et al.*, 2011)). Our result could therefore indicate that insulin secretion was more severely or earlier compromised in patients with novel frameshift mutation (c.1189 delT23in patient 3) than in patients with missense mutations leading to more severe clinical phenotypes and more often likely to use of insulin. Others studies also found a fairly high proportion of patients with more severe clinical phenotypes resulting from HNF1α frameshift mutations to use insulin treatment in most often cases (Winckler *et al.*, 2007).

The 3D structure of wild type and mutant HNF1α gene

Three-dimensional structures have been reported for the dimerization domain and the homeodomain. The dimerization domain forms a four-helix bundle, with each identical promoter contributing two α helices separated by a turn (Hua *et al.*, 2000; Rose *et al.*, 2000; Narayana *et al.*, 2001). The three α helices of the HNF-1α homeodomain are superimpose able on the three helices of other homeodomains (Fig. 1), although there is an extended loop between the second and third helices of HNF-1α relative to the canonical homeodomain fold (Ceska *et al.*, 1993; *Schott et al.*, 1997). The 3D structure of wild type and mutant HNF1α gene including missense and frameshift mutations for 8 studied patients were represented in fig. 1 (Kallberg *et al.*, 2012). The input of wild type predicted as 2 domains; domain 1 from 1-81 amino acid and its Pvalue is $6.62e^{-02}$ and domain 2 from 82-281 amino acids and its Pvalue is $4.08e^{-15}$. It is clear that mutated HNF1 proteins were different completely from wild type. Further studies should be carried out for detection the clinical aspect of these mutations.

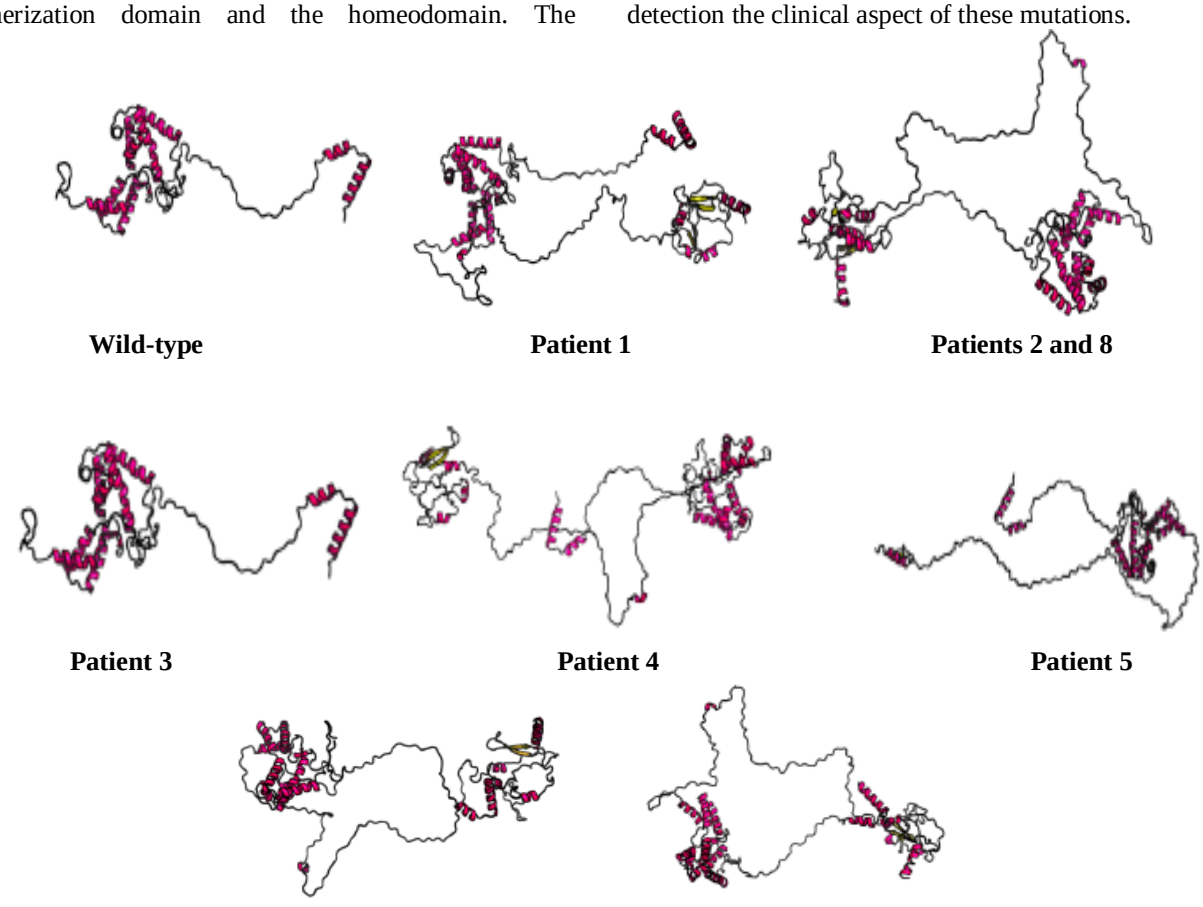

Fig 1: The 3D structure of wild type and mutant-types of protein of HNF1α (Kallberg *et al.*, 2012).

CONCLUSION

We suggest that analysing relatives together with the probands may decrease the median age at diagnosis. The position of the mutation relative to the functional domains of HNF1α not play a role in the severity of the disease. We suggest that together with these parameters other factors should be considered in other studies aiming to identify other [arameters that may influence the clinical expression of MODY3.

Certain DNA variations that could explain the hyperglycemic phenotype of the Egyptian patients. This study found variations in exonic sequences for the HNF1α gene in the patients corresponding to ten mutations. In addition to the variants I27L, E95V, E105G and S476V, which have previously been described in patients with the common form of MODY3, thereby reflecting their controversial significance variations.

Mutation detection by molecular genetic screening method is now a focus of interest in clinical medicine. Since sequencing is cost effective, hence one can use these methods to screen MODY subjects in our region, initially for exon 5 then followed by other exons of HNF1-α gene. Thus, exon 5 may expected to be the mutational hotspot in our region and may serve as a molecular marker on screening the MODY subjects. The second finding is derived from the results of the sequencing study which revealed nine novel variants all of which were identified in exon 5 before. It is mostly observed that the age at which the disease was diagnosed is around 20 - 22 years in subjects who had these polymorphisms. Therefore, the disease onset is likely to be at this age in subjects with polymorphisms in exon 5.

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